

What Bush should tell Gorbachev

The unexpected early summit meeting between the presidents of the United States and the Soviet Union could be the most fateful of its kind ever to have been held.

THE meeting in December between presidents George Bush and Mikhail Gorbachev, hastily arranged in the improbable setting of two warships off the coast of Malta, could be the most important encounter of its kind since that in 1944 at Yalta, in the Crimea, at which Stalin, Roosevelt and Churchill agreed on the partition of Europe in the mould now dramatically breaking into pieces. After a year during which the pace of change has astounded everybody, the loose ends in the arms control negotiations that need fixing will seem almost routine business. The most absorbing item on the agenda will be that of the future relationship of the Soviet Union and the East European members of the Warsaw Pact with the rest of the world. Whatever happens, the relationship has changed irreversibly. December will offer an opportunity to cement the changes of the past year and to build on them.

Gorbachev has signalled his intentions clearly enough. Earlier in the summer, when Poland elected a government with Solidarity in charge, the absence of a counter-revolution from the East could be explained by Poland's circumspect arrangement that ministries concerned with defence and internal security should remain in old hands. But the impending elections in Hungary will almost certainly bring more radical reforms, Bulgaria (with good cause) seems to be beginning on the same path while a new and presumably temporary regime in East Germany seems to have acknowledged that it cannot prevent its people from voting with their feet for the form of German reunification implied by the torrent of emigration that began in August. What most astounds the West is that all this has happened without reprisal, as in Budapest in 1956 and Prague a little later.

Popularity

Gorbachev has brought all this about by his insistence (within the Soviet Union particularly) that even Communist parties must be popular with their people, by saying that even the nations of Eastern Europe have a right to manage their own affairs and by subscribing to General de Gaulle's vision of a Europe stretching from "the Urals to the Atlantic" by his repetition of the phrase about the "common European home". It is a remarkable achievement. What can be done, and what in particular can Bush do, to cement the change? The most serious danger is that an excess of caution will mean the loss of a

great opportunity. Bush, like Gorbachev, needs a vision of what the world may be like and a plan for getting from here to there.

A first approximation to the future is that Europe will consist of independent states with borders roughly where they at present. That, by itself, would not guarantee peaceful coexistence: Germany elected Hitler to office in 1931, while the tensions between East and West Europe since 1945 have mostly arisen because elections in the East have not been free. (The constitution of the Soviet Union still assures the Communist Party a "leading role", which can be variously interpreted; until this year, the party appointed the government, but under present arrangements, the elected Supreme Soviet can veto appointments it does not like.) But all the states concerned have accepted the requirements of the Helsinki agreements reached in 1978, which guarantee such things as freedom from censorship and to travel, and which have already become a framework for military negotiations on a European scale. If supplemented by provisions requiring regular free elections by secret ballot everywhere (which concept is, admittedly, not easily defined), they would almost suffice in their present form to ensure the stability of an enlarged Europe.

Democracy

Could Bush and Gorbachev accept that as a goal? They can find out in December. Bush would be able to claim that he had brought democracy to Europe, but would have to accept that some governments would be elected communist. Gorbachev would need more breathing-space (and the Supreme Soviet will need more experience) before the leading role of the Soviet Party could be redefined. But the goal is not so distant as to be unattainable. The isolation of two warships off Malta may be just the setting in which to brood about it.

How to get there? This year's transformation of Eastern Europe points to a security problem that will become more serious as the years go by. Briefly, the Warsaw Pact needs some assurance that its formal adversary in the West, the North Atlantic Treaty Organization, will not take military advantage of changing circumstances in Eastern Europe. The negotiations already under way may see a sizeable reduction of military strength in the near future, but the Warsaw Pact needs more urgent reassurance. A non-aggression pact



would suit the need if the coinage had not been devalued, notably by the Molotov–Ribbentrop Pact of 1939. In present circumstances, an agreement finally to negotiate a German peace treaty 44 years after the event would probably do instead. A corollary would be an eventual agreement to remove national forces from foreign soil.

But economic problems will be at the front of Gorbachev's mind. This year's dramatic changes have revealed what may be their own roots — the virtual industrial bankruptcy of the Soviet bloc. It is a curious business. Shortages of consumer goods and food have been commonplace for years, but the failure of Soviet industry to modernize its processes and products has brought widespread demoralization that will not quickly be lifted even if the struggling cooperative movement (one of Gorbachev's initiatives) enjoys a fairer wind than hitherto. Nor will joint ventures with Western companies or a relaxation of the strategic embargo (which should be tried) work quickly.

Can Bush help? Not easily. Despite obvious comparisons with the post-war circumstances that evoked the Marshall Plan, President Bush has no constituency for repeating this gesture on behalf of the Soviet bloc, and in any case the generosity of the United States is constrained by the federal deficit. Nor would a huge loan from an institution such as the International Monetary Fund be of much help if the money were spent by the planners in Moscow in the same old ways. The more important need is for an institution organized like the World Bank, lending money for specified and well-defined projects, but focused on Eastern Europe and the Soviet Union. Western Europe, the chief beneficiary of the new arrangements, should stump up most of the capital. Why not?

There is also an even more urgent need — help against the privations of the winter now beginning. The Soviet Union has ten times as many people as Poland, and is at least as badly supplied with food and housing, while the means to keep warm will be put in hazard by the rash of strikes at coalmines in the past several weeks. That one common thread in the complaints of Soviet miners (who do a dirty job) is of the lack of soap is a vivid proof that the shortages are rudimentary. Can Bush do anything to help? Goodness knows. Only the Soviet government can tell. But if there is an identifiable way in which short-term credits could help, Bush should use his influence (and the creative accountancy of his budget director) to create them — and should use the opportunity to pin down Gorbachev on the pace of economic reform in the Soviet Union. Last week's tenfold devaluation of the rouble is a step in the right direction, but only a small one. (Its chief effect will be to give the Soviet treasury the benefits of the black market in foreign currency.) State-subsidized prices are a more serious distortion of reality and, for elementary reasons, the immediate causes of many of the shortages. But it would be a shame if the prospect of a more cheerful world than anybody has believed possible for half a century were dimmed for the lack of \$10,000 million or so. □

Fetal tissue wrangle

The US government's decision to continue its moratorium on fetal tissue transplants is squeamish and damages NIH.

Dr Louis Sullivan, the US Secretary of Health and Human Services, who superintends the doings of the US National Institutes of Health (NIH), has dug himself into a deep pit by deciding to continue the ban on the exploratory use with federal funds of fetal tissue transplants (see opposite). Not that the decision is surprising. The US administration, like Dr Sullivan himself, is in a cleft stick about abortion, the source of fetal tissue. It is alarmed at the unknown influence of anti-abortionists on its electoral support and also fearful that a successful appeal to the Supreme Court by anti-abortionists could land it with a bruising need to carry legislation through the Congress. So it seeks not to give offence.

The circumstances of Dr Sullivan's ban closely resemble those in which the British Prime Minister, Mrs Margaret Thatcher, a few weeks ago intervened to prevent the use of public funds for the study of British sexual behaviour. While not disputing the need for data bearing on the rate of spread of AIDS, she behaved as if public support for the project would imply public approval of the practices, some of them sexual, by which AIDS spreads. But the government said it would not object if the project were supported privately (which, thanks to the far-sightedness of the Wellcome Trust, it has been). Dr Sullivan has washed his hands of the fetal tissue issue in exactly the same illogical way, but the consequences will be serious.

It is not as if there are no precedents in the field. Earlier this year, the British Department of Health and Medical Research Council published a set of proposals for regulating work with fetal tissue that command general respect; briefly, ethical committees should give their prior consent to all proposed procedures, and money should not change hands. Anti-abortionists might complain that the rules institutionalize abortion, but are constrained from doing so by the fear of seeming to blackball what could be valuable therapeutic techniques. The truth is that the question of whether abortion should continue to be allowed, and in what circumstances, is a separate question that must be decided separately.

Meanwhile, whatever the future for the use of fetal tissue transplants in the treatment of congenital diseases, it will be disastrous if NIH cannot contribute to the research needed to find out. NIH is the only agency in the United States whose purse is long enough to support the substantial and well-controlled studies required both to explore the techniques that might be used and to determine their effectiveness. Dr Sullivan's squeamishness, while burdening charities with the need to support research and offering private hospitals a market for untried procedures, will seriously undermine the reputation of NIH for intellectual independence. □

Ban on use of fetal tissue to continue

■ Abortion politics sway the decision

■ AIDS researchers to enter the fray?

Washington

LAST week's decision by Louis Sullivan, Secretary of Health and Human Services, to continue indefinitely the moratorium on federal funding of research in which human fetal tissue from induced abortions is transplanted into human recipients was long expected, but has nonetheless aroused strong opposition.

The ban was imposed temporarily in 1988 after the National Institutes of Health (NIH) requested the assistant secretary for health's approval of an experimental implant of human fetal cells into the brain of a patient with Parkinson's disease, although approval for the experiment was not formally required. The NIH panel concluded that such research should be permitted provided safeguards were developed to prevent commercialization of fetal tissue and to separate the abortion procedure from the use of the fetal tissue. Anti-abortion activists criticized the report, arguing that its members were carefully selected by former NIH director James Wyngaarden to ensure that it would support this research. The administration rejected the advice anyway, and imposed the ban.

In a letter sent last week to Bill Raub, acting director of NIH, Sullivan stated that permitting such research would increase the incidence of abortions, and that he was "particularly convinced" by the argument that most women are equivocal about a decision to abort so that knowing that the abortion would contribute to some beneficial research might swing the decision in favour of an abortion. The NIH panel concluded that this was "highly unlikely" and could be minimized by a prohibition on discussion of the use of the fetal tissue until after the decision to abort. In the letter, Sullivan responded that such a distinction would be in practice impossible to maintain.

Clinical trials to assess the efficacy of fetal tissue in treating Parkinson's disease and juvenile diabetes are under way without federal support. But Robert Petersdorf, president of the Association of American Medical Colleges, claims that without federal funds this research will be slowed considerably, and accuses the administration of ignoring the suffering of millions who could benefit in order to accommodate a single, uncompromising segment of the community.

Predictably, Sullivan's decision was immediately welcomed by John Willke, president of the National Right to Life

Committee, and denounced by the United Parkinson Foundation, American Paralysis Association and the Juvenile Diabetes Foundation International. But a vigorous campaign against the ban is being mounted by the AIDS community, which until now has played no part in the debate. The New York-based lobbying group for AIDS patients, ACT-UP (Aids Coalition to Unleash Power), believes that research involving fetal tissue is one of the most promising fields of AIDS research and has persuaded the New York Civil Liberties Union to consider taking legal action against the administration for restricting research with potential to treat a life-threatening disease.

The enthusiasm of the AIDS community for fetal tissue research was inspired by Anthony Fauci, head of the National Institute of Allergy and Infectious Disease, who was guest speaker at an ACT-UP meeting two weeks ago. According to the transcript of the meeting, Fauci said that "fetal liver cells . . . may very well be able to completely suppress the virus and allow reconstitution [of the immune-system]". Asked whether such research might be possible while the administration is staunchly opposed to fetal tissue research, Fauci said there might have to be "some very serious pressure . . . and some real rethinking about that". Fauci could not be reached for further comment.

The House of Representatives subcommittee on health and the environment is to try to muster support in Congress for lifting the ban and will hold hearings on the issue. Subcommittee chairman Henry Waxman (Democrat - California) criticized Sullivan's decision, saying it would be "tragic" to reject the NIH panel's "sensitive and thoughtful" recommendations on the basis of abortion politics.

Christine McGourty

Signs of struggle

FREEDOM



THE US space station now has a logo, but the struggle for funds continues, page 109.

ANTARCTIC OZONE

Ozone hole heading south prematurely

Washington

THIS year's ozone loss over the Antarctic, which has already surprised many climatologists by its unexpected severity, is adding to the puzzlement by beginning to break up unusually early in the season. Last week, researchers from the National Aeronautics and Space Administration (NASA) announced that atmospheric conditions around the South Pole were becoming more active, disrupting the polar stratospheric vortex and sending volumes of ozone-poor air over the tip of South America and the Falkland Islands.

In the few years that the southern ozone hole has been recorded, an approximate two-year cycle has emerged, with odd-numbered years showing greater depletion than even-numbered. This phenomenon had been linked with another approximate two-year cycle in the Earth's atmosphere, the Quasi-Biennial Oscillation (QBO). The driving mechanism of the QBO is not known, but it is marked by a yearly reversal of the direction of stratospheric winds at mid-southern latitudes. The empirical pattern has been that, in odd years, when the QBO is in a westerly phase, the polar vortex has been stable, isolating the Antarctic air from the rest of the southern circulation, and the hole in the ozone layer has been deep.

This year, the ozone hole began to form early and quickly, as it did in 1987, and by 3 October it was apparent that the ozone depletion would be as great, and affect as large an area, as it did two years ago. But this is not the confirmation of the two-year cycle that it seems to be at first glance. Earlier this year, atmospheric scientists noted that the QBO was behaving erratically, and that the mid-latitude winds remained in an easterly phase. This led to expectations of strong atmospheric dynamics, a weak polar vortex, and a small ozone hole.

But what happened instead during the austral spring was that atmospheric dynamics remained calm despite the QBO being in its easterly phase, and the polar vortex was strong. This led to a big ozone hole, but after only a month stratospheric motions are increasing and the vortex is beginning to disperse, mixing the ozone-poor Antarctic air into the general circulation of the Southern Hemisphere.

What has broken down this year is the simplistic link between the direction of the mid-latitude winds and the strength of the polar vortex, although the early break-up may indicate a somewhat belated attempt by the stratosphere to catch up with the QBO.

Atmospheric scientists will now be looking for a set of meteorological observations to which the severity of ozone loss is more reliably correlated.

David Lindley

No evidence for neutrons at Yale/BYU

Washington

IN an interim report last week to the cold fusion advisory panel of the US Department of Energy (DoE), Moshe Gai of Yale University and Steven Jones of Brigham Young University give a preliminary analysis of the results of their joint experiments, and say that there is no evidence for any anomalous emission of neutrons from titanium chips immersed in deuterium gas.

Jones has argued for a low-level solid-state fusion phenomenon (see *Nature* 338, 737; 1989), but Gai has done similar experiments with a negative result (see *Nature* 340, 29; 1989). They agreed to collaborate on an *experimentum crucis*, completed about two months ago.

Their report to the DoE committee says they found no evidence for large bursts containing tens of neutrons, and a few examples of bursts containing half a dozen neutrons were "vetoed" by cosmic ray counters, indicating that the bursts were associated with external fluxes of cosmic ray particles rather than events in the experimental cells. But Jones does not regard the negative results as fatal. He says that the Yale/Brigham Young test was run for only 77 hours, which should be compared to an equivalent of 13,000 'measurement hours' now recorded by the experiment at Los Alamos National Laboratory in which he and Howard Menlove continue to see neutron bursts. The Los Alamos burst rate translates into only a 50 per cent expectation of a single burst from the Yale/Brigham Young set-up, he says, and one of the recorded bursts can be interpreted as a cosmic-ray event "only with some difficulty". Jones has sent a memorandum to the DoE recounting his objections. David Lindley

GLOBAL WARMING

North Sea changes CO₂ theories

London

ONLY 30 per cent of man-made carbon dioxide may be taken up by the oceans, rather than the 50 per cent predicted by existing models. This is one implication of a research programme to evaluate seasonal cycles in the North Sea, as part of the North Sea Community Project, sponsored by the UK Natural Environment Research Council (NERC). The prediction that the oceans soak up half of all man-made carbon dioxide emissions had never been rigorously tested in the field, and the discrepancy between prediction and observation has obvious implications for the study of global warming. The new results come from a study of the rate of gas exchange between the atmosphere and the surface of the sea by researchers at the Plymouth Marine Laboratory and the University of East Anglia. Henry Gee

Export curbs threaten science

Bonn

THE circus that is West German export policy offered a new attraction last week as the conservative coalition government of Helmut Kohl rushed to redraft a hastily written law meant to restrict the transfer of weapons technology to developing countries.

There was to be an emergency meeting this week to rectify the law which, to the government's embarrassment, is supported by the opposition, but opposed by its own supporters in the coalition. The law must pass the Bundestag (the lower house of parliament) on 16 November if it is to take effect by the government's self-imposed deadline of 1 January 1990.

The law was drawn up hurriedly in response to allegations earlier this year about the participation of a West German company, Imhausen-Chemie GmbH, in the building of a chemical weapons factory in Libya (see *Nature* 337, 491 and 678; 1989). The government first denied the allegations but later had to admit they were true.

Although the law is aimed principally at companies exporting equipment that could be used to produce atomic, biological or chemical weapons, it has emerged that international scientific cooperation would be one of its main victims. Cooperation would be effectively stopped by a clause requiring a government licence for the transfer of sensitive technology to countries outside the 24-member OECD (Organization for Economic Cooperation and Development). Well over a hundred countries would be affected, many of which have development agreements with West Germany that necessitate technology transfer.

Scientific organizations protested against the law before a parliamentary committee here last week. "We are hopeful but not yet confident" that the law will be changed to allow cooperation as before, said Klaus Fleischmann, executive secretary of the German National Research Centres (GFE). And the general secretary of the West German Rectors' Conference (WRK), Christian Bode, wrote to the Bundestag economics committee that the new law is a "severe threat" to the success of West German research.

In February, the government introduced regulations that require a licence for technology transfer to non-OECD countries. The new law would go a step further by setting criminal penalties — from 15 years to life imprisonment — for researchers or company employees found to have deliberately or negligently transferred technology to non-OECD countries.

What caused most alarm was a clause that made even the "negligent promotion"

of action leading to weapons manufacture a serious crime. Harald Müller of GFE argued that, for example, if an Egyptian biologist trained in West Germany were discovered years later to be working in a biological weapons plant in Libya, his West German collaborators would be subject to arrest.

Ernst-Joachim Meusel, administrative director of the Institute for Plasma Physics (IPP) in Garching, wrote in the *Frankfurter Allgemeine Zeitung* that the law was drawn up with "typical German thoroughness," which in this case was "too much of a good thing".

The threat of restrictions came at an especially inopportune time for the science organizations, which just days later celebrated the "40th anniversary of the return of German science to the international research community". In speech after speech at a gala in Bonn on 6 October, politicians and researchers dilated on the reliance of West Germany on scientific cooperation and on West Germany's "moral obligation" to help researchers in the Soviet bloc.

WRK has suggested a clause exempting scientific cooperation from the law "as far as it is not directed toward the development or production of weapons", a modification that Fleischmann supports. Conservative parliamentarians had suggested a similar clause at the hearing.

But opposition members of the Bundestag mentioned incidents that they consider serious enough to warrant the imposition of strict penalties on individual researchers, notably the case of a physicist at the IPP in Garching accused of exporting sensitive nuclear weapons technology to Pakistan (see *Nature* 338, 692; 1989). Although the science organizations tend to see such incidents as isolated cases of abuse, the Social Democrats (SPD) and Greens perceive them as part of a pattern, and complain that the GFE does not take their concerns seriously enough.

Bundestag member Albrecht Müller (SPD) said that scientists "do not seem to see atomic bomb development as a serious problem", but he admitted that threatening researchers with criminal penalties is "not a happy way out".

Even if the law is amended to protect scientific exchange, the clash with the SPD has left a bitter taste in the mouths of science organizations. Intent on making things difficult for the government, the SPD has succeeded at making researchers squirm.

Steven Dickman

Correction

THE author of the News item about drift-net fishing entitled "Japan under pressure" (*Nature* 342, 9; 1989) is Mike Shatz. □

But who is the enemy?

Washington

LAST year's renaming of the National Bureau of Standards as the National Institutes of Science and Technology (NIST) was intended as more than a cosmetic change. In its capacity as a producer of innovative, state-of-the-art devices for fundamental scientific measurement and calibration, NIST was deemed particularly suitable as a federal agency that could lead the way in turning technological research into commercial products. Thus the Advanced Technology Program (ATP) was begun earlier this year, to support joint ventures by NIST and industrial partners. But in considering continued authorization of funds for NIST, Congress has run up against the legislative puzzle of how to construct ATP so that foreign companies do not use it to gain access to US technology, trying at the same time not to hedge the programme about with so many rules and restrictions that it defeats the US companies who are meant to benefit from it.

What makes such problems additionally difficult in the United States is the general political belief that the government should not directly tell industry what to do, or hand out money to develop specific commercial products. As Representative Don Ritter (Republican-Pennsylvania) put it last week at a hearing on "What is a US company?", it has become an "article of faith" that market forces should determine the direction that industry chooses to move in. Consequently, the US government, unlike its Japanese or European counterparts, can give direction to industry only by enacting legislation that encourages some activities and discourages others.

At last week's hearing, before House of Representatives subcommittees on science, research and technology and on international scientific cooperation, the 'Japanese' point of view was put by John Kline of Georgetown University, who argued that the key to success in joint ventures was for the government side to decide clearly what the aim of a project was, and to assemble the components to achieve it. "Foreignness", Kline said, is intrinsically neither good nor bad; what mattered was whether a given company had expertise necessary for the success of the project in question. The way to do joint ventures, he suggested, was not to set general rules but to examine each case separately.

But this is an approach neither the Congress nor the administration is yet prepared to accept. Robert Cohen, an economic consultant, and Larry Hecht, of Lehigh University, both argued for a strategy along the lines of the act that set up ATP, in which rules are given for

deciding whether a company is American or foreign, and specific conditions are applied to the participation in a joint venture of a company deemed to be foreign. But, as Kline remarked, the ATP rules in their present form can exclude a foreign company from partnership for reasons that have nothing to do with the company itself, for example because of restrictive trade practices by the foreign government.

Foreignness under the ATP rules is determined simply according to the nationality of the majority of shareholders. Hecht acknowledged that this was a coarse distinction, but argued that it was the most useful definition available. The United States is the "least restrictive" of 35 countries in which he had done business, he said, but could no longer afford to be magnanimous in its treatment of foreign competitors. Hecht suggested that foreign companies should be excluded outright from any ventures in "sensitive" areas, where sensitivity is determined according to the economic as well as the military importance of a technology, and further proposed that US companies operating in such areas might be given effective legal protection from foreign takeovers.

Such stern measures were opposed by Mark Rochkind, president of the North American operation of the Dutch electronics company Philips and Neil Vander Dussen, president of the Sony Corporation of America, who pointed out that their companies, although foreign according to the ATP definition, were in effect domestic. They contributed to the US economy, carried out research in the United States, and even exported manufactured products back to their home countries. Equally, some US companies were moving their manufacturing operations into countries with lower labour rates, such as Mexico and Korea; these companies offer a route by which the results of federally-funded research can go abroad.

Although a good deal of ingenuity was spent in weighing various definitions of foreign and US companies, the debate began to seem theological. Hecht, Cohen and Kline all agreed that what was needed was some sense from the Congress or the White House of a purposeful strategy to improve technology transfer, not a series of laws with complicated rules. But in the United States even to talk of 'industrial policy' is to hint at socialism and state control.

In any case, multinational corporations around the world are moving in their own directions, and not even the US government has much real power over their choices.

David Lindley

South African ban

SOUTH Africa's Minister for Environmental Affairs, Gert Kotze, has announced that in line with the recent international decision banning all trade in ivory, the import and export of ivory through South Africa will be forbidden "at least through 1990". But Kotze also announced that South Africa will be joining the nine other African countries requesting reservations giving them permission to engage in controlled trading. South Africa has a regulated population of 8,200 elephants, and believes that a complete ban on ivory trading will undermine its successful management programme, although the country has allegedly been a poaching route for illegal ivory from other countries. M.C.

Fusion director quits

ROBERT O. Hunter, controversial director of energy research at the US Department of Energy, resigned his position on 27 October after occupying it for a little more than a year. Hunter had become increasingly embroiled in a dispute over the future of the US fusion energy programme, and would have been called soon to testify again before the House Committee on Science, Space and Technology, where he recently endured a harangue from chairman Robert Roe (Democrat, New Jersey) and others. D.L.

British quartet named

THE commercial-sector space mission Juno has announced the four final candidates from whom Britain's first astronaut will be chosen. They are Gordon Brooks, a 33-year-old physician in the Royal Navy; Major Timothy Mace of the Army Air Corps, also 33; Clive Smith, 27-year-old aerospace lecturer at Kingston Polytechnic; and Helen Sharman, aged 26, a food technologist. The four will train at the Glavcosmos facility near Moscow, and one will be chosen to join two Soviet cosmonauts in a flight in 1991. The £16 million needed to pay for the flight is to be raised by sponsorship; exactly what the sponsored astronaut will do in space has not yet been announced. □



The space woman from Mars. Helen Sharman is a research technologist with Mars Confectionery.

Dealing with the data

Washington

REPRESENTATIVES from the National Institutes of Health (NIH), the Department of Energy (DOE) and Genbank, the national sequence database at Los Alamos National Laboratory, are meeting here this week to draw up recommendations for a new interagency informatics task-force to oversee national policy on handling the data created by the mapping and sequencing of the human genome. The task-force will put proposals on both policy and technical issues before the NIH genome advisory meeting due to be held in December.

The deputy director of the NIH human genome office, Elke Jordan, warned the audience at an international conference on sequencing at Wolf Trap, Virginia, last month that unless coherent policies plans are developed now, "we are going to run into a big problem later on".

Central to the debate is the role of journals in publishing sequence data. At present Genbank shares with the European Molecular Biology Laboratory (EMBL) in Heidelberg, West Germany, responsibility for searching journals and storing genetic sequences. But Paul Gilna of Genbank says that, with the collaboration of journals, the responsibility for data entry is being shifted to the authors of the publications. An increasing number of journals are making the deposition of sequences in databanks a condition of publication.

But researchers have yet to agree on when they should be required to release data. Genbank is aiming to ensure that deposited data are available within one month of publication, but some researchers advocate delaying the public release of data until the results are refined and interpreted. James Watson, director of the genome office, has suggested that the United States restrict dissemination of its data to reserve any commercial benefit to itself (*Nature* **341**, 269; 1989). Jordan responds that widespread international collaboration makes it almost impossible to restrict dissemination of results, and says the best way to ensure reciprocity in access to data is to refuse to collaborate with those who withhold data.

Despite concern in Congress that funds for the US genome project should not subsidize foreign biotechnology industries, most researchers in the genome project are more anxious to ensure fast dissemination of results, and Jordan said that NIH would consider making it a condition of an NIH grant that the sequencing data be released at a specified time. They might also consider refusing to award grants to researchers who have delayed releasing their results.

The new task force will also, in Jordan's

words, try to find "new ways to give brownie points" for research, since the human genome project is likely to create lengthy sequences that will not be publishable in the more traditional journals. Mark Pearson, director of molecular biology at Dupont and head of the NIH informatics committee, says that researchers will increasingly include in their curriculum vitae accession numbers given to them when they deposit data in the databanks.

But with this, he warns, will come "all the perils and problems" of counting accession numbers and assigning quality weightings to them. Some researchers at the meeting balked at the suggestion that the data from their research might not merit publication. When Graham Cameron of the EMBL databank suggested that sequencing would become a routine task and that only interpretations of the data would merit publication, Sidney Brenner of the Medical Research Council in Cambridge, where researchers are sequencing the genome of the nematode, quickly retorted that if that was the case

INFECTIOUS PERESTROIKA

Bulgaria on a learning curve

London

THE three-week long 'Ecoforum' convened in Sofia, Bulgaria, as part of the CSCE (Helsinki) process ended last Friday without a final document. As at the earlier CSCE human rights meeting at Vienna, the Romanian delegation refused to agree on a text, objecting to the proposed draft on two counts.

Romania objected to a paragraph in the draft report that would have given informal groups and individual citizens the right to campaign on environmental issues, but demanded the insertion of a clause condemning the building of nuclear plants close to international boundaries — a clear reference to Bulgaria's plan to build such a plant on Belyane Island in the Danube.

According to diplomatic sources in Sofia, President Mikhail Gorbachev and President Todor Zhivkov of Bulgaria made last-minute telephone calls to the Romanian leader, Nicolas Ceaucescu, urging him to accept at least a compromise document, but in vain.

To some extent, however, the Romanian refusal was overshadowed by events taking place outside the hall. For the first time in 45 years, Bulgarian citizens were taking part in a demonstration organized at their own initiative. The demonstrators were members of Ecoglasnost, a new environmental group campaigning against proposed hydroengineering works on

Ethical matters

Paris

AT a four-day European meeting on Genetic Heritage and Human Rights held in Paris at the end of last month, a series of workshops were devoted to discussions of research on the human genome and its implications, predictive medicine, *in vitro* fertilization and biotechnology, from both a scientific and an ethical perspective. Participants formulated recommendations dealing with, among other subjects, the freezing of embryos, the psychological dangers of screening for as-yet incurable monogenetic diseases or diseases that appear only later in life, genetic 'fingerprinting', the need to conserve genetic diversity through gene banks and the existing dangers of eugenic choices. A summary of each of the 13 workshops and their recommendations will be published at the end of the year, followed at a later date by more extensive proceedings. Peter Coles

then perhaps those at the databanks would care to do the sequencing.

Christine McGourty

■ Databases have prompted correspondence this week, page 114.

Mount Rila and the river Mesta.

When Ecoglasnost was founded earlier this year, it encountered considerable harassment from the Bulgarian authorities, who refused to recognize it as a legally constituted society. During the run-up to the CSCE meeting, pressure from various embassies won the consent of the Bulgarian government to allow Ecoglasnost to organize some "fringe" activities. Nevertheless, on 26 October, there was what was later officially described as a "regrettable" incident, when the police intervened against Ecoglasnost members distributing leaflets.

The official explanation — as offered by Nikolai Dyulgerov, head of the Bulgarian CSCE delegation — is that the leaflet distributors were not in the place specified for their activities by the city authorities. But there were no arrests, Dyulgerov said; the 22 citizens concerned were simply conveyed by bus to where they should have been. Dyulgerov admitted that "the authorities had exceeded their instructions at times", but said they had been "provoked" by the conduct of some of the citizens.

The "incident", Dyulgerov said, indicates that both sides need to learn the established procedures for protest activities customary in other countries. Both sides, it seems, took his instructions to heart: and last Friday's demonstration passed off without incident. Vera Rich

Congress to abandon ship?

Washington

THE outlook for the proposed US space station Freedom looks bleak after a show-down last week between officials from the National Aeronautics and Space Administration (NASA) and members of the US House of Representatives Committee on Science, Space and Technology. The committee's support has been crucial in winning funds for the space station from a budget-conscious Congress, but now chairman Robert Roe (Democrat - New Jersey), furious at NASA over its plans to scale down the project, is threatening to propose scrapping the whole project.

Under the new plan, which was prompted by an anticipated 20 per cent cut in the space station's 1990 budget, NASA would begin construction of the space station in March 1995, but would stretch out assembly over an extra year and a half, causing delays in the schedule for attaching the European and Japanese modules. As well as delays in achieving full power capacity and in installing the full quota of crew members, there would be permanent

Nelson (Democrat-Florida) complained that there had been 11 major reviews of the space station programme in the last five years, partly because of the high turnover in management. During that time there have been four NASA administrators, five associate administrators and six programme directors.

Criticism of NASA's new plans from both the Japanese National Space Development Agency and the European Space Agency, has fuelled fears that this instability would jeopardise future international collaboration in space. Roe said that the credibility of the United States as well as the future of the space station "is on the line". A Japanese Diet member told committee member Jack Buechner (Republican - Missouri) that if the United States makes any more changes in the space station they are "going to pick up their yen and go home". But Truly maintained that the international partners were included from the beginning in formulating the new plans and that most of their complaints have been dealt with.

Further changes in the space station could also delay President Bush's proposed missions to the Moon and to Mars. If the space station is to be used as a base for developing an outpost on the Moon, it will need greater power capacity and room for more than the eight astronauts planned. Congress will be hard-pressed to provide the large increase in funds needed if NASA is to support both the Moon/Mars initiative

and the space station as they are now planned.

Minor revisions of the proposed space station are irrelevant anyway, according to Bruce Murray, head of The Planetary Society and former director of the Jet Propulsion Laboratory, who argues that it should be completely redesigned as a space port for manned flights to the Moon and Mars. Microgravity research, the primary aim of the space station at present, could be carried out on a smaller, man-tended platform serviced by shuttle flights. He is critical of the "diffused purposes, excessive costs, inefficient assembly and inadequate schedule" of the current programme and warns that if it is not changed now, the United States could repeat the disastrous inefficiencies, missed achievements and loss of international stature that were the consequence in the 1970s and 1980s of concentrating on the shuttle to the exclusion of all else.

Christine McGourty

'Merit' pay for all

Barcelona

UNIVERSITY researchers in Spain may be able to increase their salaries by up to 50 per cent by the end of their career if they can demonstrate "productivity" in teaching or research. Although this news, the consequence of a government decree made in September, has in general produced satisfaction in the universities, it infuriated researchers working for the Science Research Council (CSIC), who were excluded from the scheme and felt that they were being discriminated against. But now the CSIC has announced a similar initiative.

Salaries in Spanish universities and research institutions have always been very low compared to those in private industry, and the difference has been growing as the country's economy has improved. Compared to France, for example, salaries in Spain are similar at the beginning of the career but then progress very little, making the disparity for senior researchers very apparent. On top of this, Spain has now an annual rate of inflation of nearly 7 per cent, but salaries have increased only 3 per cent this year.

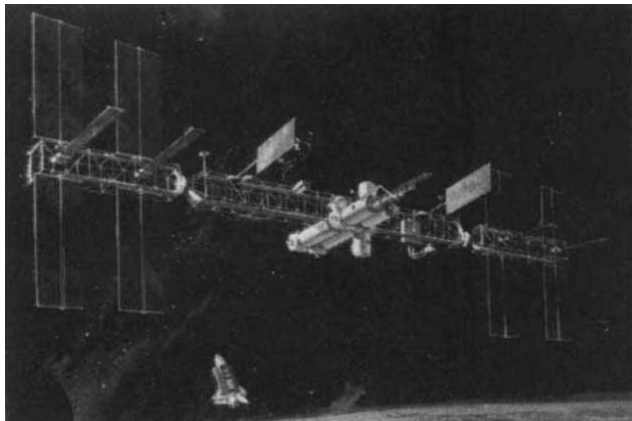
In an attempt to alleviate the problem, and to stimulate the quality of research in the universities, the government has decided to introduce a "productivity" bonus that could increase by up to 10 per cent every five years the salary of any researcher or lecturer who can show excellence in teaching or research.

Quality of teaching will be assessed by the universities, some of which have already decided to give maximum scores to everyone, and research achievement will be judged by national commissions.

Traditionally, the salaries of CSIC researchers have risen in parallel with those of university scientists, a system that led to collaboration and exchange of personnel between the two groups. But CSIC researchers were angered by not being offered a comparable productivity bonus, and feared that the omission was part of an unofficial policy to divert CSIC towards applied research and collaboration with industry. The last round of new positions created in CSIC in fact emphasized applied research, even though some of the best groups in CSIC work in basic areas of biology and physics. The controversy led to the unusual sight of scientists in laboratory coats picketing the CSIC building in Madrid.

The president of CSIC has now announced that efforts are being made to give CSIC researchers a similar career structure to their university counterparts, and to encourage further collaboration.

Pedro Puigdomenech



Vincent de Fate's artistic view of how the space station should look.

changes, such as the abandoning of plans for a new high-pressure space suit. But the associate administrator for the space station, William Lenoir, said at the hearing that many of the changes were made for technical reasons. For example, proposals for "hooks and scars" needed if the space station is to service other spacecraft have not been finalized because of insufficient technical understanding of what will be required of them. Lenoir insisted that the space station would remain essentially as planned.

Rejecting this claim, the committee accused NASA of making "draconian" changes without consulting Congress or the international partners. An irate Roe bellowed at NASA administrator Richard Truly that he would not "be made a damn fool of" by urging full financial support while NASA was reworking its plans in order to build a different low-cost, space station. The chairman of the subcommittee on space science and applications, Bill

ESF scheme criticized

London

AN initiative by the European Science Foundation (ESF) to integrate European research on schizophrenia could lead to an identification of the genetic loci associated with the disease within five years.

But reservations about the methodology and management of the scheme emerged at a discussion meeting on the molecular genetics of schizophrenia held here on 4 November. The meeting was sponsored by SANE (Schizophrenia: A National Emergency), a British charity founded in 1986.

In that same year, ESF launched a 'network' to establish a European database on pedigrees, to foster international collaboration, to create cell lines from patients and their families and above all to work out common criteria for the diagnosis of schizophrenia. At present the diagnostic criteria vary significantly from one country to another.

ESF overtures to raise the necessary FF88 million (\$14 million) came to nothing, however, apparently because the national funding agencies from whom the money was sought were not satisfied that they would have enough say in exactly how the money would be spent. The ESF has now started a FF10 million pilot programme using money that it has raised itself, which has helped to win round other funding agencies.

The ESF programme was outlined at the London meeting by Jacques Mallet, of the CNRS (Centre Nationale de la Recherche Scientifique), but two general criticisms arose. First, funding bodies are likely to be unwilling to release funds to the scheme only for the ESF to pass it on to research groups it already supports. Dr Dai Rees, Secretary of the British Medical Research Council (MRC), said he had to be sure that the MRC "isn't being charged twice".

Second, the extent to which the ESF would wish to dictate terms to laboratories participating in the scheme is unclear. Researchers are concerned that participation might compromise their independence. They worry, for example, that the search for polymorphic markers on chromosome 7 could be allotted to researchers with expertise in chromosome 11, and because they do not know to what extent ESF might license one group to perform linkage analyses between markers on data collected by others. Although Mallet gave the impression that the ESF would take full account of the preferences of individual groups, researchers and paymasters were left wanting to know a lot more before committing themselves fully.

Henry Gee

Victims of their success?

Washington

Two weeks ago, the National Science Foundation (NSF) decided not to continue to support the John von Neumann National Supercomputing Center in Princeton, New Jersey, beyond September 1990. The centre's director, Doyle Knight, now has a year to find other sources of money. The von Neumann centre's struggle for survival will be watched with concern by the other four NSF national supercomputing centres: with supercomputing capacity in the United States now eighty times greater than in 1985, when the NSF centres were begun, the provision of computing time and programming advice is no longer their sole province, and all of them are looking to justify their existence in other ways.

NSF's decision to cut off the Princeton centre caused no great surprise. It operated ETA-10 machines, whose manufacturer, Control Data Systems, got out of the supercomputer business earlier this year (see *Nature* 339, 246; 1989). The centre had proposed replacing the ETA-10s with a Cray system, but with three of the NSF centres already running Crays, the Princeton proposal was not considered sufficiently distinctive to merit support.

Knight responds that the distinctiveness of a supercomputer centre should be measured by more than "the label on the box", and points to Princeton's work in designing a comprehensible "English" interface between the supercomputer's operating language and the user as an example of the kind of pioneering development his centre was doing.

Nevertheless, Knight has no plans to fight the NSF decision, and is concentrating his energies instead on finding other means of support. These will include money from the state of New Jersey and from industrial clients as well as from academic users, but he emphasized that the von Neumann centre has no desire to become a consultancy or a time-sharing service: the technical staff now at Princeton are unlikely to stay if they have no time to work on their own research.

Although the Princeton centre has been forced into fighting for its existence, the other NSF centres are also facing challenges. Initiated at a time when supercomputer time in the United States was scarce and inaccessible, the NSF centres were meant not just to provide access but to be, as Thomas Weber, director of advanced scientific computing at the NSF, puts it, "evangelists". In that they have been successful. Nearly a dozen states have now set up their own supercomputer centres, and a number of companies that started by buying into the NSF centres have now bought their own machines.

But both Weber and Lisa Heinz, an

analyst at the Congressional Office of Technology Assessment, argue that there is still plenty for the NSF centres to do. Weber points to the fact that at least one industrial client of the National Center for Supercomputing Applications in Champaign, Illinois, has remained a client even after buying its own machine, and Heinz says that national supercomputing centres, are needed to do jobs beyond the capabilities of individual institutions. Many scientists, she says, have been so keen to get their old programmes running on the new machines that they have done the least adaptation necessary; it is mainly the NSF centre staff who have looked for the most efficient ways to solve problems on supercomputers, and who have thereby acquired the foresight to look ahead to new applications.

This view of the future is shared by Charles Bender, director of the supercomputing centre at Ohio State University in Columbus, which is supported by the state of Ohio and provides supercomputer time on a Cray Y-MP free of charge to 22 universities in the state. His centre has a staff of about 40 people and an annual budget of \$4.5 million, which makes it smaller than the NSF centres only by a factor of three or four. But last week Bender chaired a meeting in Columbus of eight directors of regional centres who together controlled more supercomputing power than the five NSF centres. He therefore sees no reason why local centres should not be capable of doing all the things that NSF centres can do now.

The Ohio facility also has links with industry (an important reason for the state's support) and staff who do research in visualization and computing methods. Bender argues that the independence of his centre from the federal budget gives it more flexibility, for example in working on the programming capabilities offered by novel devices such as the 'transputer', a parallel-processing chip made by the UK-based company Inmos, now under the wing of French company Thomson CSF.

The time from installation of a new machine to its obsolescence seems to be four or five years, which also happens to be the age of the NSF centres. Four of the five are still thriving, and Weber argues that their undisputed success in raising up US supercomputing capability from almost nothing and moving their expertise into industry is a good argument to take before Congress in asking for continued support. But he has to hope that politicians, seeing independent supercomputing centres springing up and trying to do the same thing, will not decide in a few years time that the federal effort has succeeded, and that the baton should be passed to states and to industry.

David Lindley

SUPERCOMPUTING

Cray and IBM in the frame

Sydney

AUSTRALIA's supercomputing capability is about to take a leap forward. An agreement between the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and a commercial supercomputing bureau, Leading Edge Technologies, should bring a Cray Y-MP to the country by February next year. The A\$10 million machine, which will replace an out-of-date Cyber 205 in Canberra, will be leased by CSIRO from Leading Edge and will also be available, at a cost, to industry and universities. The leasing arrangement is a consequence of an earlier miscalculation by CSIRO, which bought the Cyber 205 in 1984 in the expectation of a demand by industry. But industrial use of the machine fell far short of paying the cost.

Leading Edge, based in Melbourne, already has a Cray X-MP to which industry and academic researchers have access at A\$3,000 an hour. According to Tom Kopp, managing director of Leading Edge, the hourly charge for the Y-MP is likely to be A\$4,500.

Installation of the new machine will move the focus of Australian supercomputing from Canberra to Melbourne where, according to Bob Frater, director of the Institute for Information and Communication Technology for the CSIRO, most of the major supercomputer users reside. "Groups like the Division for Atmospheric Research and the Division of Biotechnology have, until now, been disadvantaged by having our supercomputer facilities in Canberra," he said.

The move to Melbourne will also be advantageous to the Strategic Research Foundation (SRF), an initiative of the state of Victoria to commercialize research developments by bringing academic groups such as CSIRO and the universities together with industry.

The Victorian government has promised support for the SRF to the tune of A\$3 million in 1988-89 and A\$7.5 million a year over the next four years, with additional funding "if working groups identify proposals which would have a major impact on the Victorian economy."

The Cray Y-MP reserved for CSIRO is now on the assembly line in the United States. According to Ian Elsum, the manager of the CSIRO Information and Communication Technology Institute, this has created some pressure to get the deal signed. "Cray clearly cannot reserve the machine for CSIRO indefinitely. We didn't allow enough time for something of this complexity to go through. It's a bit of a rush," he said.

Contributing to the pressure is believed to be a lack of money on CSIRO's part to buy into the deal. According to Kim Sweeney, SRF project manager, "CSIRO

doesn't have enough money to meet the cost of the Cray. They have approached us to meet the cost." SRF will discuss at 14 November board meeting whether to help CSIRO, but may invest in an IBM system.

According to sources in the SRF working party on advanced computing, IBM has approached the Victorian government with a "very competitive deal". But representatives of academic computer services groups on the SRF board favour a Cray, and a deal that linked the SRF to the CSIRO/Leading Edge Cray purchase would appease the computer buffs.

Sweeney says that people in computer advisory groups are urging SRF to look at the Cray leasing arrangement by CSIRO as the nucleus of supercomputing centre like those supported by the US National Science Foundation. SRF is willing to accommodate these desires "in one way or another".

Tania Ewing

FRENCH RESEARCH

Fifty and counting for CNRS

Paris

FRANCE's chief basic research organization, the Centre National de la Recherche Scientifique (CNRS), celebrated its fiftieth anniversary this October in the presence of President François Mitterrand — the first visit in its history from the head of state. Although it would be wrong to say CNRS is lying about its age — the organization was officially founded on 19 October 1939 — it was only in 1945, after the Second World War and the end of the Nazi occupation, that CNRS took shape. Since then, it has grown to be Europe's largest basic research employer, with 26,000 staff, 17,000 of whom are researchers and technicians, and 1,300 research laboratories.

The size and centralized administration of CNRS have attracted criticism, and successive governments have attempted reforms. In 1986, Jacques Chirac's neo-gaullist government tried to dismantle what it called an unwieldy and unmanageable "mastodon" in an effort to return research and prestige to the universities. But the moves ended in the resignation of the research minister and riots in which a student died.

Although CNRS is relatively safe in the hands of the present socialist government, research minister Hubert Curien is shortly to announce measures that will change the way the organization is run. A former director-general of CNRS, Curien intends to limit the director-general's hand in the choice of heads of the 45 disciplines and to demand a drastic cut in the number of administrative disciplines. Peter Coles

ENERGY GENERATION

Six per cent of solution

London

THE UK Department of Energy is considering proposals to build a barrage in the Severn estuary, at the southern junction of England and Wales, which could supply up to 6 per cent of Britain's future electricity needs. In a report published last month, the Severn Tidal Power Group and a consortium of civil engineers conclude that the scheme is feasible, and that "the level of confidence in the project, its buildability, programming and cost estimation have been considerably enhanced".

The plan is to erect a 10-mile barrage across the Severn river between Brean Down, west of Weston-Super-Mare, to Lavernock Point near Cardiff, where the tidal range reaches 35 feet, its peak value along the estuary. The report, which cost £4.26 million, estimates that construction of the barrage could begin in 1993 with the first electricity being produced by 1999.

The estimated cost of the barrage is £8,280 million at 1988 prices. There would be 216 turbines built into the wall which, given the Severn's unusually large intertidal range, could produce up to 7,200 megawatts — between 6 and 7 per cent of Britain's annual electricity requirement.



The turbines would be stationed at a depth of 100 feet in large hydraulic structures or caissons. The barrage, largest of a number of sites being considered by the Department of Energy, could be a source of clean energy for 100 years.

The report warns, however, that there would be environmental effects that need further study. But if the barrage replaced an equivalent coal- or oil-based electricity-generating capacity, there would be a 17.6 million tonne reduction in carbon-dioxide emission, according to the report. The Department of the Environment is also assessing the barrage's potential for recreational activities and tourism.

The report concludes that the "the Severn Barrage remains the largest single renewable energy project which could make a significant contribution to the electrical energy supply on a reasonable timescale".

Ben Webb

An end to patronage?

London

THE East German Academy of Sciences last week issued a statement calling for appointments to management posts in science, education, the health service and the economy to be made on merit, not by Party allegiance. In what amounts to a rejection of the *nomenklatura* system, the Presidium of the Academy declared that posts should be filled according to the professional competence, leadership qualities, moral integrity and "solidarity with socialism" of the candidate. The meaning of this last phrase was not made entirely clear, but the term was explicitly contrasted with "Party membership", whose value as a "decisive criterion" was rejected. Moreover, the Academy said, all elective posts in these fields should be made by secret ballot, and from a genuine choice of candidates.

The Presidium statement maintained that, in the past, political decision makers had largely ignored the research results and expert studies produced by Academy scholars. Scientists, it said, have a decisive role to play in the "spiritual and material renewal" of East Germany, and a fresh strategy for science must therefore form part of the much-needed reform programme; there should be a transition to "nonbureaucratic" forms of planning which would promote initiative. To give science the status it deserves, the Academy proposed that a special

parliamentary science committee should be established, while existing scientific management and advisory bodies should be reduced in number but made more efficient. Finally, the Presidium put in a not-too-discreet hint about funding. "We are strongly opposed", it said, "to producing prestigious goods for show and to diverting large reserves from scientifically and economically important tasks." At a time when East German society is in turmoil, the statement tries to balance conservatism and reform. On the one hand, it said that it expected "a considerable contribution" to ending the crisis to be made by the forthcoming meeting of

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

East Germany's exodus continues: jubilant refugees, mainly Trabant drivers, arrive in Schirnding in the West. (AP).

the General Committee of the Socialist Unity Party. On the other hand, it declared its support for "new social solutions, and the open debate of opinions".

Reasoned scientific analysis, the drawing up of expert opinions, and "responsible disciplined work" are the Academy's prescription for the ills of East Germany.

Vera Rich

HUNGARIAN SCIENCE

Pay rise move to stem brain drain

London

IN an attempt to halt Hungary's brain-drain, Prime Minister Miklos Nemeth has promised the country's 25,000 research workers in science, education and health a pay raise of 16 per cent over and above the rate of inflation. Since the beginning of this year, up to ten scientists a day have been leaving the country to take up research posts abroad. The latest figures indicate that some 12 per cent of Hungary's scientists take posts abroad each year, and one quarter of these stay away for more than five years, or take up permanent residence in the West.

Professional frustration is as much a cause as low salaries. Many scientists find it impossible to do their experimental work in Hungary, and hence ask their foreign colleagues for short-term invitations to work abroad.

Hungary's lack of Western currency makes it difficult to buy advanced labora-

tory equipment, but the main problem is the way in which science is financed. For decades, the Hungarian budget has been divided up according to the "residual principle" of old-style Marxism: "non-productive" activities — science, education, and health — were given what remained in the coffers after all other expenses had been met.

During 1987 and 1988, and particularly after the establishment of the independent Democratic Trade Union of Scientific Workers (TUDDOSZ) in April 1988, Hungarian scientists became increasingly vocal in their criticism of this principle. In a letter to the Hungarian Academy of Sciences announcing the pay rise, Premier Nemeth has now promised that the residual principle would be replaced by a system in which the allocation of funding to science, education and health will reflect the importance of these areas.

Vera Rich

Cycle nears biggest peak

Washington

As solar activity increases towards a projected 11-year maximum in February next year, the peak of the current solar cycle is on a course to challenge the 1958 maximum as the biggest on record. Already, two solar flares of unprecedented magnitude have occurred this year, disrupting radio communications and in one case setting off safety cut-outs in the electricity supply grid in Quebec and triggering a blackout over most of the province.

Solar activity is conventionally measured by the number of sunspots, and for cycle 22, now approaching its peak, the number is running level with the record of cycle 19, which peaked about 33 years ago. But because the 11-year periodicity in the solar cycle is only approximate, whether cycle 22 exceeds cycle 19 will depend on whether the sunspot number keeps on rising as projected or begins to decline a little earlier than expected. In terms of terrestrial disturbances, the sunspot number is in any case somewhat academic; what causes trouble is magnetic activity, including solar flares, which tends to peak a year or two after the sunspot maximum and whose severity is imperfectly correlated with the sunspot number.

The two large flares this year, one occurring in March and the other last week, were both of a size and intensity that Joe Allen, director of solar-terrestrial physics for the National Oceanic and Atmosphere Administration (NOAA) in Boulder, Colorado, said he had not previously seen. The flares produced intense X-ray emission, measured by NOAA satellites, and more unusually had been observed in ground-based neutron and proton detectors.

One indication of the severity of this year's events, according to Allen, was that it was becoming increasingly difficult to track the thousands of mostly unidentified objects orbiting the Earth. Magnetic disturbances propagating from the Sun cause local electromagnetic disturbances which heat the atmosphere by generating currents. The visible consequence is more aurorae — there have been reports from as far south as the Mexican border — but for orbiting bodies the important effect is that the heated atmosphere expands away from the Earth a little, increasing frictional drag. Typically, in the tens of thousands of orbits tracked by the US Department of Defense, about a thousand every day change enough to make identification with a previous orbit impossible. But soon after the giant solar flares, said Allen, the number of lost orbits went up to 6,000 a day.

David Lindley

Harmony among physicists

SIR—I would like to comment on the article entitled “Is the end of particle proliferation at hand?” (*Nature* 341, 555; 1989), which gives a distorted and alarming picture about how scientific research proceeds and progresses.

True, the scientific importance of the results announced about the measurements of the Z^0 -width both at SLAC and at CERN is presented to readers, but at the same time — and quite unnecessarily in my opinion — there is an added element of rivalry and hostility.

Your article also gives a totally distorted view of my own “reactions” to this important result, based solely on some statements I am said to have made to the Italian newspaper *La Repubblica*. In fact I have never granted such an interview to the author of the article, Arnaldo d’Amico. He has based his “story” on patched up, “secondhand” information from local news agencies which have twisted the meaning of my words on the occasion of a scientific talk in Pisa. This has been fully clarified in a letter to the editor of *La Repubblica*, published on 20 October. Finally, even in your “translation” from Italian into English, there are some significant exaggerations.

I am surprised that a journal of the scientific reputation of *Nature* did not verify the authenticity of the story (for instance checking what I said with me directly) before going to press. The article would then have lost much added spice, but at the same time would have gained objectivity.

The article could also use some brushing up when it comes to scientific matters. The statement that LEP, by virtue of its higher beam energy, has produced more Z^0 s than SLC is simply shocking: the beams of the two machines must have *identical* energies in order to tune on the Z^0 . I also fail to see what is meant by the statement that “the lack of a clear answer [on the exact number of additional families] has impeded theoretical understanding”. Finally, contrary to what is said in the article, the top quark is not the only particle “awaiting discovery” — the tau-neutrino has also so far escaped direct detection.

It is a great pity that the two announcements of excellent results from both machines have been described mainly as an episode of acrimony and rivalry among scientists, whereas in fact I believe that it is their convergence that is the significant and durable fact.

We all know that, nowadays, fundamental research takes place simultaneously at several laboratories in the world, with the common, ultimate aim of arriving at accurate and confirmed results. In this process, competition is certainly healthy and stimulating, but it rests on the implicit

understanding that science is thus best served. Advantage should not be taken to provoke “public quarrels”, not even by reporters.

CARLO RUBBIA

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■ *Nature* did seek confirmation of Dr Rubbia’s reported remarks in advance of publication, but was told by his office at CERN that he was away and inaccessible. — Editor, *Nature*.

Attention please

SIR—Stephen Jay Gould seems to be both right and wrong in his defence of the word ‘attende’ to describe someone who attends a meeting (*Nature* 340, 424; 1989). He is no doubt right to say that the *Oxford English Dictionary* recognizes the use of the word, but he is surely wrong in assuming that such recognition implies formal approval as correct English. In the absence of a formally constituted governing body, it is probably impossible to state that certain constructions are correct or incorrect for all time. All that we can hope for from a dictionary of English is guidance to current usage; beyond that we simply have to aim for a reasonable level of consistency to avoid confusion. Certainly Americans may use ‘attende’ to mean one who attends, but to do so is to mix up the object of the verb in the usual form of construction (such as examinee, one who is examined) with the subject (examiner, the one examining). If you insist upon doing so, and can get enough people to accept your usage, then a dictionary will no doubt in time reflect that fact, but the language as a tool for communication will suffer. English is a difficult enough language as it is because of its loose structure, but that is no reason to throw out such guidelines as we do have and confer the status of neologisms on mistakes.

By the way, in the spirit of Gould’s reply to David Pyke and if one wishes to assess correctness on the basis of usage, it is simply not true to assert that ‘attender’ “has never been used to mean ‘one who attends a meeting’”. The *Concise Oxford Dictionary of Current English* (1982) gives ‘attender’ as the noun for one who attends in the sense, among others, of “be present at (lecture, etc.)”. So where is the linguistic need claimed by Gould?

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South African aid?

SIR—I hold no special brief for the views expressed by File *et al.* (*Nature* 341, 96–98; 1989) in their article “Towards the day of hard choices” about education in South Africa, but I believe it behoves all scientists to get out their calculators and do a few sums on the figures they quote.

Table 1 gives the per capita expenditure on white education in 1987 as R2,508 and on black as R477. The number of scholars for the same year is given as 954,000 white and 6,645,000 black. From these numbers, expenditure on education in 1987 can be calculated to be $R5.6 \times 10^9$. Education is said to consume “a respectable 20 per cent of public expenditure”.

If in 1987 expenditure on black education had been at the same per capita rate as for whites (and I doubt whether the expenditure on white scholars would count as very generous by standards elsewhere in the West), the cost would have been 3.4 times greater at $R1.9 \times 10^{10}$, equal to 68 per cent of public expenditure.

Education, as stated by File *et al.*, is in competition with other much-needed public expenditure, whatever savings might arise from the elimination of *apartheid*. Direct taxation is not low by international standards and there is a general sales tax of 13 per cent. Government, moreover, is committed to trying to contain public expenditure in order to control inflation.

To spread the 1987 budget evenly raises the expenditure for blacks by 50 per cent to R732, but will barely scratch the surface of the problem of providing an adequate system for all. To bring the 1987 school population to white levels of expenditure over 5 or 10 years would require a real per annum compound growth in the education budget of 28 per cent or 13 per cent respectively — by an economy currently experiencing a growth rate of 1–2 per cent.

File *et al.* quote projections suggesting that by the year 2020 the school population will be 899,000 whites and 14,977,000 blacks. If they are all to receive education equivalent to that of whites today, the cost at 1987 prices will be $R4 \times 10^{10}$ — a seven-fold increase over 1987 and requiring a real per annum compound growth of expenditure of 6 per cent.

I feel confident, if not happy, in predicting that such increases will not be achieved unless there is massive international aid, either to education directly or to boost the economy so that it can itself generate the necessary wealth. The “hard choices” are not really South Africa’s — there are too few resources to allow that luxury — but the outside world’s: whether to help or not.

K. L. MANCHESTER

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Benefits of databases

SIR—I am appalled by the comments of John Maddox (*Nature* 341, 277; 1989) on databases, which contain a pot-pourri of excuses for inaction. While consistent policies for dealing with databanks are desirable, the variation in quality, subject matter and stages of development makes it inappropriate to consider them all collectively. Each must be judged on its merits. I am concerned with the DNA sequence databases.

The real benefits of the DNA databases will come when they are available promptly, so that a researcher can read a journal and immediately access a sequence whose properties are described. This can be achieved only if the sequence is present in the database before publication. Indeed, as Maddox points out, for a manuscript describing a sequence to be reviewed properly, the original data must be available. Moreover, a sequence in typewritten form is of little value, since even the simplest search requires a computer. It is nonsense to suggest that Indian scientists would be discriminated against if sequence submission to databases were mandatory before publication. No one, whether from East or West, can make proper use of a sequence without an electronic version and a program to assist in its analysis. A computer is every bit as necessary as a centrifuge to today's molecular biologist. Should we relax our requirements for data from scientists with only limited access to centrifuges?

The remarks about the Soviet Union are puzzling. The key issue is not where the data are collected, but whether the data deposited in the databanks are available to everybody, which they are. A scientist in the Soviet Union or India or the United States has essentially equal access to the data, by subscribing to a databank and receiving the data on a regular basis.

The thorny issue of industry versus academy is a red herring. The requirements for publication of sequence papers should be the same for both academic and industrial authors. Methods should be described in sufficient detail so that another researcher can reproduce them. The data underlying the conclusions drawn must similarly be available to the readers. If the manuscript describes a sequence, then it is imperative that the sequence be available. If industry wishes to keep its sequences secret, then it should not publish. Worries about the improper commercial use of nucleotide sequence data seem unfounded. If programs in the public domain are not sufficient to allow analysis of the sequences, then, of course, companies should seize the opportunity and sell software that is capable of that analysis. This is the essence of a free-

enterprise system. It must not be a reason for failing to make the databanks as comprehensive as possible.

Scientific journals depend for their existence upon the scientists who perform research just as much as scientists depend upon the journals to promulgate their results. So far, this symbiosis has been a healthy one. However, times are changing. Computers are here to stay and soon journals themselves must be available in electronic form. Equally, the data encapsulated within each scientific article need to be presented in ways that the scientific community can understand and digest. The present system worked well when there were only a few scientists and fewer journals. But electronic databases are now an integral component of the scientific enterprise. Scientists and journal editors have a responsibility to steer the publication process through its next evolutionary stage. A natural and appropriate step in this process will be for the journals and their contributors to join together in ensuring that the sequence databases become the timely resource that they should be. It is a simple matter to require that the author of a sequence paper provides a database accession number for that sequence as a prerequisite to publication. Indeed, this is the only point in the process at which it can be enforced and it is appropriate to do so. To require the granting agencies to police this scheme is unwieldy and impractical. Only when authors cheat by inventing accession numbers should the granting agencies become involved.

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SIR—The staff of the Protein Data Bank (PDB) read with great interest "Making good databanks better".

The PDB, an archival database for three-dimensional structures of biological macromolecules, was established at Brookhaven National Laboratory in 1971. With financial support from the US National Science Foundation, the National Institutes of Health and the Department of Energy, the PDB is compiled and made available to a broad user community worldwide. Currently, the PDB is distributing data for more than 450 structures, with about 140 additional structures in the process of being input. The data input process includes extensive checking by PDB staff, with feedback to depositors allowing for the correction of errors. PDB depositors and users alike find that this quality control constitutes a valuable service.

There is a wide variation in the policy of scientific journals regarding the deposi-

tion of crystallographic data, and the PDB relies to a substantial degree upon voluntary contributions. However, as indicated in the article, journals have a right to require that essential supporting data accompany manuscripts submitted for publication and should also arrange to provide access to these data. In the case of crystallographic studies of macromolecules, the only practical means to provide such access is by submission of the data to a database, since the information (atomic coordinates and structure factors) is far too voluminous to be useful in hard-copy form. In recognition of the above considerations, the Commission on Biological Macromolecules of the International Union of Crystallography has recently endorsed a policy that publications should be accompanied by deposition of the appropriate data in the PDB. The policy provides the option for a delay in the release of the deposited data of up to one year from the date of publication for coordinates and up to four years for structure factors, reflecting concern that results from the early stages of analysis will be inaccurate in detail and that investigators should have the opportunity to complete the analysis and interpretation of their data.

Nature should reconsider its policy of not requiring deposition of data in the appropriate databases. In addition to ensuring maximum availability of the information to the scientific community, archiving by the databases removes the very real possibility that essential information will become lost over time.

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Reversed charges

SIR—One method to slow rising journal costs (*Nature* 341, 349–350; 1989) is to put more of the publication costs on the authors, because they receive the major benefits of their publications. Many learned societies already have page charges and lower subscription costs than commercially published journals. Page charges should be standard for all journals because publication costs are a legitimate part of a research project. Publication is the final and most important step in a research project as it satisfies the 'publish or perish' directive and places the data in the scientific record.

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■ There are strong objections to page charges in international journals whose contributors work in varied circumstances—Editor, *Nature*. □

Vaccines for the Third World

Barry R. Bloom

New vaccines, developed through genetic engineering, can make immunization an even more effective weapon for tackling disease in developing countries. So what is preventing progress?

Where there is no vision, the people perish
... Proverbs 29:18

THE Third World is the place where three-quarters of the people of this planet live, where 86 per cent of all births and 96 per cent of child and infant deaths occur^{1,2}. At both a national and a human level the diverse problems of the developing countries are of staggering proportions. Most have heavy foreign debt and, as a consequence, these countries now transfer to the developed countries more hard currency than they receive (\$31.1 billion in 1987)³. Most are burdened by disease (Table 1, see over)⁴; the reality is that millions of people are sick because they are poor, and poorer because they are sick. Some indicators of the quality of life of people of the 40 poorest countries are listed in Table 2 (see over), and the trends have not been encouraging. Per capita income has declined over the past five years and the percentage of national budgets spent on health has been unchanged or has diminished for eight years.

Yet one aspect of life there has improved profoundly. The number of children receiving immunizations has risen from 5 per cent in 1974 to over 60 per cent this year. The World Health Organization Expanded Programme for Immunization (EPI) prevented the deaths of 2.2 million children last year. Through the efforts of 25,000 professional national and international staff and hundreds of thousands of field workers, 60 million children are now vaccinated annually against diphtheria, pertussis, tetanus (DPT), polio, measles and tuberculosis. The number of cases of paralytic poliomyelitis has declined in the Americas from 4,500 ten years ago to fewer than 200 this year, and WHO has just made the eradication of polio one of its goals⁵. Immunization is the most cost-effective weapon for disease prevention in developing countries, and new molecular and genetic technologies are making new types of vaccines feasible. The eradication of smallpox demonstrated that they can be effective everywhere. What is lacking is the will to make the advances of modern biomedical science available to the poorest people of the world.

Vaccine problems and constraints. As impressive as the results of global childhood immunization have been, there are many diseases for which vaccines are not

available, as well as inherent limitations in each of the currently used vaccines. Only two EPI vaccines — BCG, used to immunize against tuberculosis, and oral polio vaccine — can be given at birth or any time thereafter, and BCG and measles are the only vaccines that require a single immunizing dose. DPT and polio must be given

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This was smallpox, a disease that has been eradicated through immunization.

several times to achieve protective levels of antibodies. Because maternal antibodies circulating in the infant inactivate the vaccines, DPT can be given only at six weeks of age, and then at monthly intervals up to 14 weeks. The dropout rate is high — about 20 per cent fail to return for each required booster shot. In the case of the vaccine against measles, which is responsible for the death of 2 million children annually in the developing world, the presence of maternal antibodies that neutralize the vaccine in young children represents a 'wall' that scientists have not yet been able to hurdle. If measles vaccine were given to children in developing countries at 12–15 months, the time recommended in the developed world, up to 30–50 per cent would have contracted the disease before receiving the vaccine. Measles vaccine is given at 9–12 months and a new strain may even be effective at 6 months. In all, a total of five contacts are required between the child and the health services, which represents a considerable logistical problem.

Any new or improved vaccine to be considered for inclusion in the EPI should, ideally, have the following attributes: (1) it must be inexpensive; (2) it must be safe; (3) it must be extremely effective, inducing protection in 90–100 per cent of recipients; (4) it should engender lifetime immunity; (5) it should be heat-stable and not need to be kept cold at all stages (a cold chain), which is both expensive and subject to catastrophe; (6) it should require only one shot or be compatible with the schedule for other vaccines; (7) it should be simple to give, and because of the problems of reuse of needles and of HIV infection it should be given by a non-invasive route; and (8) it should be capable of being given as close to birth as possible. Dr W. Foege, Chairman of the Task Force on Child Survival, has described the ideal vaccine as one in which "single administration at birth will provide protection from multiple diseases".

New vaccines from old. There are basically four kinds of vaccine, each of which has strengths and limitations for use in the Third World.

- **Killed vaccines** are among the simplest and least expensive to prepare. They contain many antigens and assure some responsiveness in virtually all individuals. But even under defined production conditions, some vary in reproducibility, and all require careful monitoring to assure that no viable organisms are present. As in the case of pertussis, killed vaccines often have a higher level of reactogenicity or toxicity than is desirable.

- **Subunit vaccines** prepared from individual components of a pathogen, by chemical synthesis or recombinant DNA technology (for example tetanus and diphtheria toxoids) have several advantages. They are chemically defined, reproducibly prepared and assayed, and are usually inexpensive to manufacture. Carbohydrate antigens are important for protection against many pathogens. Because complex carbohydrates cannot be produced by recombinant DNA technology, subunit vaccines, particularly carbohydrate-carrier-protein conjugate vaccines, are the only feasible strategy at present for producing protective immunity to pathogens such as *Hemophilus influenzae* b and *Streptococcus pneumoniae*, which cause meningitis, mental retardation and pneumonia. ▶

A general drawback of both killed and subunit vaccines is the need for several immunizations and boosters — immunological memory benefits from repeated stimulation by antigens. Some chemically defined subunit or peptide vaccines contain a desired antigenic determinant (epitope) that elicits B-cell-produced neutralizing antibodies, but they may not engender immunological memory to the infectious agent which requires expansion of specific T-helper cells as well; thus reinfection will not result in a boosting of the immune response. The possible use of small synthetic epitopes in subunit vaccines raises the concern that polyclonal antibodies developed against a single epitope of a pathogen may function very much like monoclonal antibodies and select for escape mutants — that is, antigenic mutants with altered epitopes on neutralizing antigens that are not neutralized by existing antibodies in the population. Although this would not be catastrophic for a single epitope in the first instance, new antigenic mutants could be created that would, in time, accumulate and possibly become resistant to normal acquired immune defences.

• *Live attenuated vaccines* have largely been derived by empirical methods, and those in current use have the advantages of generally inexpensive production, persisting immunity and a good safety record. Nevertheless, there are considerable problems with existing live vaccines in terms of reversion to virulence (polio, Sabin type 3), reactogenicity (BCG), need for a cold chain, and possible induction of disease in immunodeficient and some normal individuals (for example polio).

• *Anti-idiotype vaccines* are a novel concept that has yet to be realized. They are based on the principle that because antibody active sites (the idiotypic) are complementary to the specific antigenic determinant to which they bind, some antibodies raised against a particular antibody active site may indeed mimic antigen. Although immunization has been achieved in some experimental systems with anti-idiotype or anti-antibody vaccines, so far there has been difficulty in achieving high levels of immunization and immunological memory because T cells are not developed against antigens of the pathogen. It remains to be seen in what circumstances anti-idiotype vaccines will prove to be useful.

In considering the various criteria for new vaccines for the Third World, genetically engineering live attenuated vaccines to become multivaccine vectors that can immunize simultaneously against multiple antigens is particularly appealing. Two basic strategies are being developed, one using viral vaccines, the other using bacterial vaccines as recombinant multivaccine vehicles. Viral vaccines in general

TABLE 1 Diseases of the Third World that potentially could be prevented by vaccines*

Condition	Deaths per year (million)	Estimated episodes or incidence per year (million)
Respiratory disease	10	15
Diarrhoea	4.3	28
Tuberculosis	3 [†]	10
Measles	2	67
Malaria	1.5	150
Hepatitis B	0.8	3.7
Meningitis	0.35	1
Schistosomiasis	0.33	10
AIDS	0.1 [‡]	0.75
Worm infections	<0.06	4,900

* Modified from J.A. Walsh (ref. 4).

† Data from WHO Tuberculosis Unit.

‡ Data from WHO, unpublished estimates.

TABLE 2 Indicators of the quality of life in the world's 40 poorest countries, 1988

Average annual gross national product per capita	\$270
Literacy	
Males	42%
Females	21%
Population with access to water	
Urban	61%
Rural	21%
Government expenditure	
Health	5.5%
Education	14%
Defence	13.8%
Population per physician with access to health services	6,050 40%
Life expectancy at birth	48 yr
Infant mortality (<1yr per 1,000 births)	130
Mortality rate of children aged under 5 (per 1,000 births)	211
Population annual growth rate (1973–84)	2.6%
Children suffering malnutrition	31%
Children immunized with DPT, polio, measles and BCG	61%

have certain advantages: they express antigens in eukaryotic cells with correct folding, proteolytic processing, glycosylation, secretion and subunit assembly; and they can stimulate the production of cytotoxic T-lymphocytes as well as antibodies. Bacterial vaccines have a special ability to immunize for long periods and engender local immunity, for example in the gut, and cell-mediated immunity.

The technology: the power of molecular biology and genetics. The ability to change living organisms by genetic engineering has given rise to the possibility of devising vaccine vehicles that can immunize simultaneously against antigens of

different pathogens. Such multivaccine vehicles can be developed from either viral or bacterial vaccines, and each approach has its particular advantages.

• *Live attenuated viral vaccine vehicles.* Smallpox was eradicated by the use of live attenuated strains of vaccinia virus, originally derived by Jenner from a cowpox virus. Although vaccinia may be of somewhat dubious provenance, it has been astoundingly effective. The virus is a large double-stranded DNA virus of about 185,000 base pairs that contains all the information for its own transcription and replication in the cytoplasm of a wide range of host cells. Large amounts of DNA, approximately 25,000 base pairs, are expendable and not required for vaccinia replication. The molecular strategy to develop vaccinia into a multivaccine vehicle is based on the ability to replace non-essential viral DNA with foreign genes, and have them expressed under the control of the vaccinia-virus promoters and transcription system. Plasmids have been constructed containing one or more promoters, cloning sites for introducing DNA for foreign antigen genes, and a selectable marker, all flanked by DNA sequences that complement segments of non-essential DNA of the virus. When cells containing the plasmid are infected with vaccinia virus, a low frequency of double recombinations can occur between the flanking DNA sequences surrounding the foreign DNA in the plasmid and the non-essential DNA of the virus, resulting in the precise replacement of the viral DNA sequences by the desired promoter and foreign antigen gene. A wide range of viral, bacterial and protozoal antigens has been expressed in vaccinia, as many as four antigens being expressed simultaneously in a single recombinant virus⁶.

The principal disadvantage of vaccinia is the frequency of complications, which approaches the limits of current acceptability. Although only 1 in 300,000 recipients of the least troublesome vaccinia strain developed severe neurological effects, the incidence of serious complications, including disseminated vaccinia and vaccinia gangrenosum, was of the order of 1 in 1,000 in New York State, and is certainly higher in some developing countries⁷. (It is regrettable that data on complications in developing countries are not available from the global eradication campaign.) By genetic engineering it should be possible to define viral genes involved in neurovirulence and reactogenicity and to create new, less reactogenic, vaccine strains. It has also been possible to incorporate genes for lymphokines such as interleukin-2 to enhance immune responses to foreign antigens and the virus itself.

Because recipients produce neutralizing antibodies to the virus, a second

limitation of vaccinia is that it is not feasible, at least in the short term, to give booster shots, because reintroduced recombinant vaccinia will simply be neutralized. Practically speaking, vaccinia is a one-shot vaccine by necessity, and that shot must be highly effective to make it useful. Recombinant vaccinia expressing the glycoprotein antigen of rabies virus is extraordinarily immunogenic and, despite some uncertainty about vaccinia's acceptability in human beings, recombinant vaccinia has recently been introduced in Belgium, and a region of France, in bait to protect foxes against rabies. More importantly, perhaps, there is an epidemic in West Africa of rinderpest, a cattle disease caused by a measles-like morbillavirus, and the International Commission of Epizootics plans to immunize hundreds of millions of cattle with recombinant vaccinia expressing rinderpest antigens. In an experiment of that scale, with perhaps 100,000 vaccinators, there is bound to be adventitious infection of human beings with the recombinant vaccine. It will be essential that they are pre-immunized against vaccinia virus.

Adenovirus vaccines have been used in tens of millions of US military recruits to protect against respiratory disease, essentially without serious complications. Curiously, the vaccine is not an attenuated strain, but rather consists of two virulent strains, types 4 and 7, that are given by an unnatural route, orally, that immunizes without producing disease. The use of enteric-coated lyophilized vaccine tablets represents a distinct advantage for delivery. The virus has a rather small DNA genome, but a limited number of foreign genes have been introduced and expressed in adenoviruses by a similar, precise gene-replacement strategy, including the hepatitis-B surface antigen that is not well expressed in vaccinia⁸. Concerns include the role of the *E1A* gene, which functions like certain proto-oncogenes in transformation of cells in culture, and the fact that the safety of existing adenovirus vaccines in children is unknown. Herpes viruses are large DNA viruses that, like vaccinia, have large amounts of expendable DNA; they could be developed into recombinant multi-vaccine vehicles, provided that the genes for neurovirulence can be identified and deleted.

Two RNA viruses offer promise as recombinant vaccine vectors. The live attenuated polio vaccine is one of the most effective vaccines in the developed world, but it has been disappointingly ineffective in many developing countries with up to 30 per cent of recipients failing to produce acceptable neutralizing titres, generally to type 3. There has been a return to multiple injections of killed polio vaccine in some places. Polio vaccines are not without problems, even in

the developed countries, because essentially all cases of poliomyelitis there result from reversion of vaccine strains to virulence. Because Sabin type 3 vaccine differs from virulent poliovirus type 3 by only two mutations, reversion to virulence is an intrinsic problem.

The key to the genetic manipulation of polio was the discovery that artificially produced complementary DNA to the viral RNA is infectious and, on transfection of cells, produces infectious poliovirus. Of the several neutralizing epitopes on polioviruses, all but one are conformational and composed of discontinuous sequences of amino acids, the exception being a single loop of linear sequence⁹. Several laboratories have been able to recombine cDNA sequences of the loop that generate the main neutralizing epitopes, for example, from type 3 into the very stable polio type 1 vaccine, to create hybrid vaccines that generate neutralizing antibodies to both types 1 and 3 poliovirus¹⁰⁻¹². The cDNA sequences encoding the loop can be modified to express sequences for small epitopes of foreign antigens, including HIV. At present, however, there is no evidence that these recombinant vaccines can immunize by oral administration.

One of the safest and most effective live attenuated viral vaccines known is the 17D vaccine against yellow fever. A single immunization results in sustained neutralizing titres for periods of over 40 years, and complications are very rare. It has recently been possible to produce infectious cDNA from the 17D vaccine¹³ and one hopes that the possibility of developing 17D as a recombinant vaccine vector will be rapidly explored.

• *Live attenuated bacterial vaccine vehicles.* The first modern approach to a rationally designed live attenuated vaccine was the production, by chemical mutagenesis, of an auxotrophic mutant of the virulent bacterium *Salmonella typhi*. A mutant in galactose epimerase (*GalE*) was selected that could not convert UDP-galactose to UDP-glucose. This strain, Ty21a, retained the ability to infect after oral administration and to colonize the gut, essential for producing secretory immunity. The mutation was designed to be a time-bomb; Ty21a can grow for several days, and then, when it accumulates more UDP-galactose than it can tolerate, it dies, liberating antigens. This strain has proven to be remarkably stable and safe, and has been tested in field trials in Egypt and Chile against typhoid fever.

Ty21a suffers from two disadvantages. One never knows precisely what gene(s) is affected by chemical mutagenesis. Indeed, when the *GalE* gene from *S. typhi* was deleted by another group and the deleted strain was tested in volunteers, two out of the four recipients developed typhoid fever, revealing that the key

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

The campaign against smallpox continued even after the last endemic case. This poster advertises a \$1,000 reward for anyone reporting an occurrence.

attenuating mutation in Ty21a must have been in a gene other than *GalE*. Equally distressing, three oral immunizations of Ty21a were required to achieve 67 per cent protection, an efficiency insufficient for a useful anti-typhoid vaccine or multi-vaccine vehicle.

To avoid the vagaries of chemical mutagenesis, the genetic strategy for salmonella vectors is to delete genes required for pathogenesis or survival, then to insert foreign genes into the chromosome by gene replacement, or express them on plasmids in the cytoplasm. A promising set of targets for gene deletion has been genes (*aroA*, *C* and *D*) of the aromatic-amino-acid pathway required for synthesis of folate and enterochelins (iron-binding proteins) as well as proteins¹⁴. These are auxotrophic mutants that cannot grow without added aromatic amino acids, and thus they too function as time-bombs. Vast amounts of foreign genetic material can be introduced and expressed in bacteria, and these salmonella auxotrophic mutants have immunized animals against introduced recombinant antigens.

A second genetic strategy used to produce recombinant *Salmonella typhimurium* vectors has been deletion of the genes for adenyl cyclase (*cya*) and the cAMP-binding protein (*crp* — catabolite repressor protein) which, together with removal of a plasmid containing virulence genes, has eliminated pathogenicity. Genes for foreign antigens expressed on plasmids introduced into these strains have persisted and immunized mice¹⁵. The great advantage of salmonella recombinant vectors is that they can be given orally, and, in experimental animals, they immunize both locally and, at the T-cell level, systemically. ►

Unfortunately, the few trials in human volunteers with recombinant salmonella vectors have indicated that the strains tested have been overattenuated and do not produce adequate secretory IgA antibodies. Patient efforts are required to construct salmonella strains that can achieve the delicate balance between appropriate attenuation and effective colonization leading to immunization. They must immunize not only against typhoid but against introduced recombinant antigens; of particular interest are those for cholera, shigella and enterotoxigenic *E. coli*.

The oldest and most widely used live attenuated vaccine in the world is, perhaps surprisingly, BCG — *Bacille Calmette-Guerin* — an attenuated bovine tubercle bacillus used to immunize against tuberculosis. This mycobacterial vaccine has been given to over 1.5 billion people with a low frequency of serious complications, although it has the highest rate of minor reactions of any of the EPI vaccines. Its effectiveness in protecting against pulmonary tuberculosis has varied greatly in different parts of the world, but BCG requires only a single shot to engender cell-mediated immunity for periods of 5–50 years. It is known to be an effective adjuvant, enhancing immune responses to many different antigens. BCG can be administered at birth or at any time thereafter, and can be given repeatedly. Like most bacterial vaccines, it is very inexpensive (\$0.055 per dose).

In contrast to *E. coli*, BCG grows very slowly and requires about 24 days to produce a colony (*E. coli* takes 8 hours). Because it seemed unlikely that direct genetic manipulation of mycobacteria would prove efficient, a 'shuttle' strategy has been devised to introduce and express foreign genes in BCG. Basically, phages or plasmids were constructed that can replicate both in *E. coli* and in mycobacteria¹⁶. Foreign genes and selectable markers can be introduced by standard molecular genetic techniques in *E. coli*, and the recombinant DNA is then 'shuttled' into BCG. Foreign genes can then be expressed either by replacing non-essential BCG chromosomal genes or from multicopy plasmids in the cytoplasm. It remains to be seen, however, how effective antigens known to be immunogenic when mixed with mycobacterial adjuvants are when expressed by the organism itself.

The spectre of AIDS. There is a serious risk with any live vaccine, namely dissemination and serious complications in immunodeficient individuals. The current HIV epidemic in many countries heightens concern about side effects from childhood vaccination. For example, vaccinia has been administered to US military personnel, 28 of whom were later found to be

The logistical problem — an immunization team in the Philippines sets out by canoe.

HIV seropositive. One died of generalized vaccinia. (One cannot help but wonder, as, by convention, study or use of smallpox (variola) virus has been discontinued and all strains presumably locked away in three safe repositories, why anyone in the world is being vaccinated against smallpox.) Several major cohort studies are underway in Africa to evaluate the consequences of standard childhood vaccination in HIV-seropositive compared to HIV-seronegative children. It is encouraging that so far no discernible differences in untoward complications of any of the vaccines have been observed. It may be that there is a window in time in congenitally infected children during which they can be immunized and before which any significant immunodepression occurs. Because children who will be immunocompromised are even more susceptible to the principal childhood diseases, particularly measles, it is strongly recommended by WHO that these children receive all the childhood vaccines, except for BCG in children with symptomatic immunodeficiency.

The basic premise of existing vaccines has long been the utilitarian principle of providing the greatest good for the greatest number of people; it has long been recognized that small numbers of individuals will suffer severe complications. At the very least, antidotes to restrict disseminated infection of recombinant live vaccines should be available (for example, vaccinia immune globulin, isoniazid for BCG, antibiotics for salmonella). Because of the high cost of drugs potentially useful against AIDS, it is sadly likely that most will not be available to patients in developing countries in the foreseeable future; consideration of cost effectiveness

alone should spur efforts to develop effective vaccines against AIDS.

Single-dose, controlled-release vaccines.

The requirement for several injections is the main limitation to subunit vaccines. Almost 800,000 neonates per year still die of tetanus, which can be prevented simply by immunizing women of childbearing age. Controlled-release systems are already being used in man (and cattle) to deliver an array of drugs and hormones. Some of these systems have been used by the WHO Special Programme on Human Reproduction (HRP), which has supported research on anti-fertility vaccines. Taking advantage of HRP expertise in the area, development of single-dose vaccines has become a goal of the WHO Special Programme for Vaccine Development with neonatal tetanus as its first target.

Basically, polymers of lactic and glycolic acids (PLA/PGA) approved for human use are being incorporated into controlled-release microspheres or microcapsules which can provide either continuous release of tetanus toxoid over a period of several months, or a pulsed release similar to booster shots, that would occur, for example, 4 and 8 weeks after a single immunization. Should this prove effective, DPT, and a number of other subunit antigens could be incorporated into this type of one-shot vaccine — not the least important of which will be epitopes from human chorionic gonadotropin or sperm to control fertility¹⁷.

Immunological problems. At a scientific level, the constraints on development of new vaccines are not likely to come from molecular biology and genetics; rather they will be biological and immunological.

IMAGE
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Although antigens have been cloned from virtually every known pathogen, and many T and B epitopes have been mapped on various antigens, there are very few pathogens for which the mechanism of protection is understood. Nor is it clear which antigens are required to engender those protective responses.

A multitude of unanswered questions remain. Must a protective vaccine against malaria sporozoites generate antibodies, or T cells, or both? Can production of specific immunoglobulin isotypes be targeted (for example IgA for intestinal pathogens, IgE for killing schistosomes, IgG2 for carbohydrate antigens)? Can the type of T cells (for example helper T-cells, cytotoxic T-cells, gamma-delta T-cells, and lymphokines) produced be controlled (for example by targeting the vaccine to the cytoplasmic compartment or endosomal compartments of antigen-presenting cells)? Can T-cell responses against important protective antigens be generated in individuals of all histocompatibility types? Can nonvarying epitopes be found on highly polymorphic protective antigens that can be effective against genetic variants of the pathogens (for example malaria, HIV, African trypanosomes)?

At the same time, immunology offers novel solutions for basic vaccine problems. Measles vaccine cannot be used in children under the age of 6–9 months because it is inactivated by maternal antibodies, but it is conceivable that appropriate measles T-cell epitopes could be given at birth in one of the recombinant vaccine vectors, such that T-cell memory is generated to measles and a protective response would follow natural infection. Clearly, the easy vaccines have already been made; new vaccines pose greater challenges for research.

'It is only a matter of implementation.' There are few more portentous words than these to be found in any health document. For most scientists engaged in the development of new health interventions, the fulfilment of their research is a product that goes through clinical testing and is eventually licensed for use. In international health, the historical record belies such optimism. For example, all of the vaccines in the EPI programme were available before 1974, but only 5 per cent of the world's children received them — it was clearly only a matter of implementation.

Certainly, the main limiting factor is cost, but it is not the only one. The scientific infrastructure for evaluating new drugs and vaccines relevant to Third World diseases is also limiting. It is difficult and expensive to carry out clinical trials in industrialized countries in which the diseases do not occur. From the beginning of the WHO-Tropical Disease

Research (TDR) programme a component was mandated in addition to the scientific research programmes for "institutional research strengthening" in the countries worst afflicted. This support, representing 25 per cent of the budget, has been used to set up and modernize laboratories, and to train bright students abroad (and, occasionally, even to make it sufficiently attractive for them to return home). As a consequence, in many developing countries there are laboratories that are able to tackle tropical diseases.

What has been largely overlooked, however, is the role of field and epidemiological research in developing countries. Almost all incentives — financial, working conditions and personal recognition — motivate people to go into medical practice, laboratory-based research or, most commonly, administration. There are few rewards for health workers in the field. Yet the field worker is the mainstay for acquiring information about local health problems, for evaluating new interventions and for integrating, maintaining and monitoring them in control programmes. Recognition of their importance through the development of appropriate career structures and educational and material incentives is a major challenge to the developing countries.

At the same time, the development of new drugs and vaccines (38 new products that have resulted from the WHO-TDR programme are currently in field trials) provides a unique opportunity for building scientific and field infrastructures in the Third World. Field-research and control infrastructures need no longer be tied directly to one drug or vaccine, but can be continuing mechanisms to assess different health strategies and disease-control activities. Much of the same technology — enzyme-linked immunosorbent assays (ELISA) or polymerase chain reactions (PCR), for example — can be used in the same place to assess the distribution of parasites in mosquitos and the incidence of multiple infections by multiple pathogens in the population. The epidemiological tools for testing different drugs against one disease are often applicable to trials against another and can be adapted to evaluating vector control and vaccines as well. The result of this research is not papers; it is the control of disease.

A vital aspect of implementation has almost invariably been ignored. Fifteen years ago it became clear that although oral polio vaccine is superbly effective in developed countries, it was proving disappointing in developing countries. Studies show that only 40–80 per cent of children receiving oral polio vaccine produce antibodies sufficient to ensure protection. To this day, it is unclear why oral polio vaccine is so ineffective in developing countries. Is it that children are already colonized with enteric viruses

that have a competitive advantage over the vaccine, or that maternal antibodies neutralize the vaccine? Or is it simply that the vaccine sold in the United States contains six times more viral particles?

Until two years ago, EPI's mandate was 'implementation' only, and it lacked a research component. The tragedy, measured in lost lives, has been misapprehension that there can be implementation without research. In this context, I mean not only laboratory-based research, but social and economic research to provide information on costs and benefits, and to provide the means to understand how best to design and implement control programmes and identify and overcome obstacles to their effectiveness.

The main trend in development since the Second World War, as the Nobel prize-winning economist Theodore Schultz pointed out¹⁸, has been investment in physical capital — dams, factories and so on — with little recognition of the potential for investment in 'human capital'. The role of health, education and quality of life in development has generally been underestimated, because it is much easier to measure direct industrial and agricultural productivity than the indirect political and economic effects of health and knowledge on the economies or political stability of developing countries.

Vaccines are clearly not the key to development. Yet they can and do serve as an entry point both to strengthening science and primary health-care infrastructures. The infrastructure for vaccines can be extended to other low-cost/high-impact medical technologies on a mass scale, including oral rehydration, and maternal and child care and education, especially breastfeeding, food supplementation and family planning. In 1984, for example, Colombia vaccinated three-quarters of its children under five years of age through three national immunization days, and has subsequently mobilized complementary efforts for primary health care and education.

Much ink has been spilled on 'appropriate technology' for the Third World, but there has been almost no consideration of 'appropriate science'. Research on vaccines involves a knowledge of molecular biology, genetics, immunology and epidemiology, and could serve as a stimulus to the educational and scientific advancement important to development. Even the poorest countries have need for expertise and access to biomedical science; they cannot afford to squander their resources on iron lungs.

The bottom line. It is ultimately, one supposes, a matter of priorities. The annual budget for the entire World Health Organization is \$327 million. The TDR Programme for six tropical diseases has a

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Out of the laboratory and into practice — announcement of an immunization day in Bolivia.

budget of \$30 million, and the budget for the Special Programme for Vaccine Development has a budget of \$2.8 million. The annual cost of childhood immunization is some \$500 million, averaging approximately \$10 per immunized child. The vaccines themselves represent less than 10 per cent of the cost of immunization; of the remaining costs for personnel, transport and logistics, 70 per cent is borne by the countries themselves. The major advance in vaccine coverage has been accomplished largely through the combined efforts of the EPI of WHO, UNICEF, The World Bank, and the UN Development Programme, with support from non-governmental organizations, particularly the Rockefeller Foundation, Rotary International and the Save the Children Fund, which together coordinate their efforts through the Task Force for Child Survival. Behind the scenes scientists trying to develop new or improved vaccines scramble for support, most of it coming from the US National Institute for Allergy and Infectious Diseases or the Department of Defense, or from WHO's Special (extrabudgetary) Programmes for Research and Training in Tropical Diseases, Human Reproduction, or Vaccine Development. A decade ago, some foundations were generous contributors to research on diseases of the Third World, but their interest seems more to have reflected fashion than a serious commitment to the problem. And one must add that, regrettably, few Third World leaders have made health a high priority.

There has been only limited interest by the private sector in development of vaccines for the industrialized world, and virtually none in vaccines for the Third World¹⁹. The reasons are simple. Vaccines account for only 1 per cent of the profits of the pharmaceutical industry, and a greater percentage of their liability. One of the two new vaccines licensed in the past ten years is a subunit vaccine against *Hemophilus influenza* b, the main cause of meningitis and mental retardation in

young children around the world. It consists simply of a bacterial carbohydrate conjugated to tetanus or diphtheria toxoids to augment the immunogenicity. The cost for each of the companies trying to develop that vaccine has been \$8–12 million. The cost would almost certainly be higher for live vaccine vectors.

Another example: hepatitis-B virus is not only the cause of a serious infectious disease, but the principal known risk factor for hepatocellular carcinoma, which is particularly prevalent in Asia. Several vaccines are currently being produced, one of which is available to EPI for large-scale public health use at \$1 per dose. Nevertheless it cannot be included in EPI because of cost. Because of the numbers of people involved, there *must* be incentives to develop and deliver vaccines and essential drugs to the Third World. It should at least be possible to establish cost-plus agreements with international agencies to develop potentially useful interventions, or joint ventures or affiliations in developing countries.

The imagination and resources of the private sector that were so instrumental in developing biotechnology should be engaged, and the private sector may find that it has something both to contribute and to gain. Failing that, developing countries that can afford it will have to rely on their own abilities, intellectual and material, for developing their own biotechnology. This is already happening in Cuba, India, Brazil and Mexico. It is important to note that several new vaccines now in field trials have, in fact, been developed by scientists in the Third World — vaccines against leprosy (developed in Venezuela and India), leishmaniasis (Venezuela and Brazil) and dengue haemorrhagic fever (Thailand). Finally, Third World countries have enormous foreign debt, much of which will clearly have to be written off. If even a small portion of that debt were restructured to be spent in local currency for health and education, several of the problems affect-

ing the quality of life in the Third World could be addressed.

The First World is the place where 13 per cent of the world's people consume the major part of the planet's crude resources, most of which come from the Third World. In the United States we spend annually over \$300 billion on 'defence'; a single B-2 'stealth' bomber costs \$532 million. In terms of personal consumption, \$55 billion (\$287 per adult) is spent annually on alcohol, \$38 billion on tobacco and \$22 billion on toys²⁰. We can afford to do more for health in the Third World. Conscience should motivate us to do so, and self-interest supports the claims of conscience. In the first place, good public health translates into good economics. We save over \$0.5 billion per year in no longer having to control smallpox (the global figure is \$2.5 billion). Second, in our world there is nothing which is truly remote and no one from whom we are disconnected. AIDS has again demonstrated that; another example, dengue haemorrhagic fever, which has been ravaging the Caribbean, and for which an effective vaccine does not yet exist, is predicted to spread in epidemic proportions to parts of the United States. Finally, it is becoming more and more clear that poverty and disease have not only a moral impact but a political price. Ultimately, what is the cost of political turmoil? We have the resources to make vaccines and essential drugs available to the people of the Third World; what we need are the imagination and the will. □

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Are electrons mere billiard-balls?

Electrons near the top of an electronic band appear to behave at low temperatures as if they were classical particles and not particles best represented by quantum mechanics.

Eindhoven

ALL of us have found ourselves on whistle-stop tours of other people's laboratories — fifteen minutes for each laboratory, but, because the visit begins twenty minutes late, nobody quite knows whether there is any time in which to talk. So it was last week at the Dutch research laboratory of Philips, the multinational company that does not object if it is called an "electronics giant", which used to be the case before the Sonys and Hitachis of this world stole the show in the hardware shops.

One stumbles into a spanking clean laboratory with a clever cryostat in the corner, one of half a dozen shining faces begins describing how they have together worked out a technique for explaining that the motion of electrons can be accounted for as if they were billiard balls even in circumstances in which quantum theory should apply (the cryostat is not irrelevant). Rashly, one volunteers that there is a paper on just that topic in the latest issue (23 October) of *Physical Review Letters* and, foolishly (because unnecessarily) one adds "from somewhere like Bell Labs". In unison they cry, "But that's *our* paper!" (The reference is 63, 1857; 1989).

What follows is more than a monument to that fleeting embarrassment, because the underlying issues are both interesting and important. One of the objectives of electronic device development is the further reduction of the physical dimensions of the circuit elements that conduct, store or change the phase of (usually to delay) alternating currents (resistors, capacitors and inductors respectively).

Recent technology, notably the development of techniques for making, by epitaxial growth, alternating layers of materials such as GaAs and its alloy with aluminium (AlGaAs), each of which may be given a predetermined shape, have made it possible to make measurements of essentially one-dimensional conductors in which the chemical potential (in simple language, the availability) of electrons can be varied at will. People would like to have a simple picture in their heads to describe the behaviour of electrons in these circumstances.

The place to start is not with quantum mechanics but with a model of what may be expected of the behaviour of an electron-sea in a reasonably well-conducting semiconductor which may, or may not, be immersed in a uniform magnetic field. It is also easy to imagine that electrons may be

injected (by the application of a voltage) from one layer in an epitaxial sandwich into the next, for example through a small aperture a fraction of a micrometre across (250 nanometres seems fashionable). The injected electrons will come from the top of a Fermi band in the layer of material that constitutes the source. They may be injected into a material whose band structure is different, but in general will have an energy that does not coincide with one of those allowed for the material in which they find themselves. How, then, will they move?

One way of dealing with it is to use the states accessible to electrons as representative of a complete set of wave functions in terms of which the motion of the injected electron may be described. It would be a straightforward if complicated calculation, especially if there were a magnetic field. Curiously, there seems to be a simpler way. C. W. J. Beenakker and his colleagues at the Philips Laboratory have followed others with an especially neat way of studying the behaviour of electrons in situations such as these.

Two years or so ago, they began with the simple objective of measuring the conductance of electrons through a single gateway between one epitaxial layer and the next. The conductance is a function both of the geometry of the gate and of its voltage. What most clearly emerges is that, at liquid helium temperatures, the conductance increases with the voltage in equal steps, each of which is numerically equal to $e^2/\pi h$ (B. J. van Wees *et al.* *Phys. Rev. Lett.* 60, 848; 1988). The conductance, it appears, is quantized.

The explanation may not be far to seek, although it has not been pinned down. The electrons injected from one region into another have energy corresponding to the Fermi energy, that of a filled band in one section of the material. It is possible to represent the transmission from one region to the next by the properties of the wave function of electrons at the top of a filled band, and then to calculate the likelihood of transmission through the gate by averaging over all states near the Fermi surface. Early last year, van Wees and colleagues at Philips were claiming that this process could yield the particular quantization of conductance observed, but the point remains to be confirmed.

Now, the group has moved on to a more sophisticated way of studying the transport of electrons near the top of the Fermi

surface in materials such as GaAs. Following others, but with more sophisticated small-scale fabrication, they have built two gates into the boundary between one layer of GaAs and another, using one as a source of electrons and the other as a detector. In these circumstances, the effect of a magnetic field on the electrons can be followed, even at the temperature of liquid helium or a fraction of it. The technique seems to have been developed by the Soviet physicist V. S. Tsui as a means of studying experimentally the behaviour of electrons in materials in which the mean free path of electrons is relatively high micrometres).

If one believed in simple quantum mechanics, one would guess that the chance that an electron leaving the gate would be deflected towards the detector by a transverse magnetic field would be a smooth but fluctuating function of the field strength. A field that would just suffice to deflect a classical electron from the gate to the detector would probably yield the maximum current, but neighbouring values of the field would carry almost equal numbers of electrons to the detector.

But again, it seems, there is a simpler way. What the Philips group appears to have found is that the electrons can be thought to behave ballistically, as if they were billiard balls. At least at sufficiently low temperatures, the chance that there will be a large current at the detector depends critically on the magnetic field, and is greatest when the magnetic field is such that the distance between the emitter and the collector is an integral multiple of the cyclotron radius of an electron in the applied magnetic field. Again, it seems, the electrons behave ballistically.

Nobody pretends, on evidence such as this, that quantum mechanics is a pack of lies, or that it is always safe and proper to regard electrons as little billiard balls. After all, the electrons are not moving in a vacuum but in the electric field of complicated lattice structures where they are denied a natural place. No doubt, it is only a matter of time before their apparently ballistic character is related to the properties of the wave functions of electrons such as these. But it is a fair guess that, when that is done, many other curious magnetoresistive properties with a quantized structure, the quantum Hall effect among them, will be more easily understood than at present.

John Maddox

Water storage in the mantle

Thomas J. Ahrens

THE origins of the oceans and Earth's volatile atmosphere are major unsolved problems in earth sciences. The recent study by Finger *et al.*¹ of the crystal chemistry of a water-bearing mineral phase, cryptically named² phase B and grown under conditions equivalent to a depth in the Earth of 360 km, dramatically demonstrates that several times the Earth's visible water budget could be stored in the mantle.

It seems now that there exists a whole family of water-bearing ferro-magnesium-silicate mantle minerals. Small samples of several of these have been recognized in natural (mantle-derived) rocks and/or prepared in the laboratory under mantle conditions. Phase B is the most dense and deep-seated of these yet discovered (see table). The work of Finger *et al.* establishes the crystallographic details of this relatively complex phase and a newly synthesized related anhydrous high-pressure phase. These new hydrous and anhydrous phases contain silicon in both four- and sixfold coordination with oxygen. This observation is particularly

interesting because silicate minerals of the crust and upper mantle from depths down to 400 km (for example, olivine Mg_2SiO_4) have silicon in fourfold coordination with oxygen. In contrast, possible minerals, such as MgSiO_3 (perovskite)³, of the lower mantle (below a depth of 670 km) have silicon in sixfold coordination with oxygen. It is therefore logical that the stability field of phase B is in the so-called transition region of the mantle (see figure). In 1986, Liu⁴ discovered that phase B breaks down to one or more phases at pressures above 22 gigapascal (see figure). Thus we might anticipate that future high-pressure research will yet uncover what phase(s) may contain water in the lower mantle. We may also anticipate that these phase(s) will contain silicon in complete sixfold coordination with oxygen such as is found in stishovite and MgSiO_3 (perovskite).

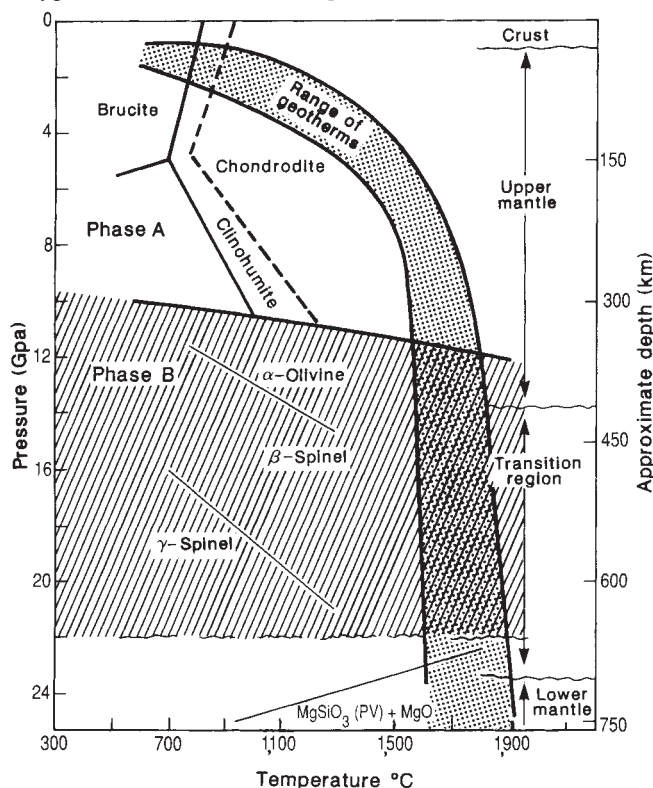
If one first looks at plate tectonics to see whether substantial masses of water can be injected into the mantle via processes such as the subduction that take place beneath oceanic trenches, the answer seems to be emphatically "no". The thermodynamics of subduction of a crust containing water-bearing oozes, silt and sand sediment overlying basalts and gabbros (containing water tied up chemically in such minerals as prehnite, brucite, serpentine, chlorite, epidote, talc and various amphiboles) was studied in detail by Anderson, Uyeda and Miyashiro⁵. They showed that a subducting plate completely dehydrates or partially melts upon being thrust into the mantle and the water-rich melt rises. Moreover, they demonstrated how this dehydration gives rise to the formation of volcanic arc rocks above

the subducting slab. Also, a substantial decrease in heat flow is observed across virtually all the oceanic crustal sections of subducting slabs as a result of dehydration reactions.

Nevertheless, current ideas about how the Earth formed — through the aggregation of smaller bodies in the young Solar System — suggest that a considerable amount of water is bound up in its interior. Much of this water may be the remnant of

Water-bearing phases in the Earth's mantle

Phase	Composition	Density (g cm^{-3})	Mass (per cent) H_2O
Serpentine	$\text{Mg}_3\text{Si}_2\text{O}_9\text{H}_4$	2.55	12.99
Brucite	$\text{Mg}(\text{OH})_2$	2.37	30.86
Phase A	$\text{Mg}_7\text{Si}_2\text{O}_{14}\text{H}_6$	2.96	11.8
Chondrodite	$\text{Mg}_5\text{Si}_2\text{O}_{10}\text{H}_2$	3.06	5.3
Clinohumite	$\text{Mg}_9\text{Si}_4\text{O}_{18}\text{H}_2$	3.14	2.9
Phase B	$\text{Mg}_{12}\text{Si}_4\text{O}_{21}\text{H}_2$	3.37	2.4



Approximate stability fields of water-bearing minerals (see table) in the pressure (and depth in the Earth) versus temperature plane. A range of geotherms for the Earth, and the stability ranges of the olivine-stoichiometry phases in the mantle, are indicated by α -olivine, β -spinel, and γ -spinel, and MgSiO_3 (PV) + MgO fields. The upper mantle is dominated by olivine. The transition region of the Earth is dominated by β -spinel and γ -spinel, whereas the lower mantle of the Earth is shown to be predominantly MgSiO_3 (perovskite) + MgO (periclase). (After Akimoto and Akaogi¹¹.)

the earliest phases of the Earth's accretion. At what stages as the Earth grew could water have been retained in the mantle in such phases as chondrodite, clinohumite, phase A and, now, phase B? In my view⁶, there are essentially two modes of Earth accretion: mode A, whereby infall and accretion of planetesimals occurs on a relatively cool Earth encased in an atmosphere nearly transparent to infrared radiation; and mode B, which gives rise to the generation of an infrared-opaque blanketing atmosphere of the type described by Abe and Matsui⁷. This mode B atmosphere, containing CO , CO_2 , and H_2O , traps the energy of impact and prevents it from being radiated away into space.

The blanketing atmosphere is expected to overlie a magma ocean. The magma ocean may be in chemical equilibrium with the atmosphere. Growth of the Earth appears to have started out in mode A — the infalling planetesimals had speeds roughly equal to the escape velocity, sufficiently low for the impacts to vaporize little of either the proto-Earth or the bolide. Laboratory experiments^{8,9} demonstrate that when the Earth's radius was somewhere in the range 500–1,400 km, impacts started to vaporize the H_2O , CO_2 , CH_4 , NH_3 and noble gases associated with the solid phases of the planet-forming planetesimals. Thus, we can define an initial primitive accretion core of the Earth with a mass of around 7×10^{25} g of material that did not lose a substantial amount of gas.

The primitive core is presumably the source of the flux of ^3He that is observed to be currently degassing (at a rate of 4 ± 1 atoms $\text{cm}^{-2} \text{s}^{-1}$) from the mantle. One can demonstrate that if the ^3He content of this core is assumed to equal that of primitive carbonaceous chondrites, the observed

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flux of ^3He from the deep mantle could be sustained over the age of the Earth (4.6×10^9 years). Primitive meteorites such as carbonaceous chondrites have not been appreciably heated since the Solar System formed (4.6×10^9 years ago) and have a similar composition to the Sun: they are considered by many planetary scientists to be a valid compositional model of the Earth-forming planetesimals.

Once volatilization upon impact occurred, depending upon the rate of accretion, the Earth probably produced a magma ocean. The magma ocean existed either until the time that the accretion rate decreased or until the Earth sustained a massive collision (with perhaps a Moon- or even a Mars-sized object with an energy of up to 10^{36} ergs) that could strip the proto-Earth of its massive proto-atmosphere. It is not clear how much water was retained during the magma-ocean phase, mode B, of Earth accretion. The Earth continued to accrete material (and still does) at a slow rate after the magma-ocean phase. We are thus back to a mode A condition.

Recently, Schmitt *et al.*¹⁰ suggested, on the basis of the excess content of siderophile elements (those preferring the metal phases) in the mantle, that approximately 0.7 per cent by weight of the Earth accreted in the late-stage mode (A). If this material contained 3 per cent water, which is reasonable for planetesimals, the entire Earth's oceans could have been derived from it. Similarly, some 2 per cent of the Earth's primitive accretion core would store an ocean-full (1.4×10^{24} g) of water deep in the Earth's interior. How much water the Earth's mantle retained during the magma-ocean phase of accretion is hard to constrain, although Abe and Matsui⁷ suggest that at least one more ocean of water was added to the Earth during this period. Thus, plausible water budgets for the Earth's interior (mantle) are in the range of at least two oceans' worth. □

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Burying the hatchet

Paul G. Bahn

THE first international congress* on the reburial of human remains that have been unearthed by archaeologists, which took place recently, was followed by the symbolic reinterment of some prehistoric bones of Native Americans near the site of the famous massacre at Wounded Knee, South Dakota. With this, the two sides in the reburial debate, the archaeologists and the native populations, symbolically buried the hatchet.

As a result of recent furore over reburial, few museums now display human remains and several collections have already been returned. In Australia, the Crowther collection of Tasmanian remains was handed over and cremated in

sympathetic to the indigenous cause and native peoples willing to accept some analysis of remains before reburial. But the extreme indigenous view, that permanent curation of any human remains is totally unacceptable, was also expressed vociferously. Several native Americans even found pictures or videos of skeletal remains to be offensive, and there was an objection to burial grounds being described as archaeological sites. Some emotive speeches were made by native American elders, most notably William Tallbull, a Cheyenne, who spoke of the spirits' journey having been disrupted, and of the need to correct this wrong. Others referred to desecration, and to the ancestors being



Peter Newark's Western Americana

Burial of the dead after the Battle of Wounded Knee, South Dakota, 29 December 1890.

1985, and recently the extensive Murray Black collection of 1,800 skeletons has been returned to aboriginal communities. In America, following the return to local tribes of entire collections by Stanford University (*Nature* **340**, 9; 1989) and by the University of Minnesota, the Smithsonian Institution, whose collection of over 18,000 native American remains and thousands of grave goods is by far the largest in the country, has recently reached a tentative compromise with Indian leaders, which allows the return of any remains and artefacts that can be linked 'with reasonable certainty' to present-day tribes (*Nature* **341**, 175; 1989).

Participants at the congress represented primarily the middle ground — scientists

imprisoned and needing release.

The archaeologists generally failed to explain adequately their need to study funerary remains. In the past, few have bothered to make their findings accessible to indigenous communities, who therefore see no benefit whatsoever in having their ancestors disturbed and removed. Only recently have efforts been made, with great success in Australia, North America and elsewhere, to involve local people in archaeological projects and provide them with clear reports.

In archaeological terms there has been a dramatic overnight transformation in a discipline which, previously, rarely considered the ethics of its practices. To some extent, the congress was already outdated, as the battle has been fought and won: most archaeologists and anthropologists now accept that indigenous peoples have a

* The First Inter-Congress of the World Archaeological Congress Archaeological Ethics and the Treatment of the Dead, Vermillion, South Dakota, 7–10 August 1989.

strong moral case, and have no objection to the reburial of recent skeletal remains. All but the extremists, who unfortunately did not attend the congress to put the case for rejecting the whole notion of reburial of what they consider to be a priceless scientific resource, probably now concur that reburial may be carried out after a period of study. Laws have been passed in 22 US states against disturbing Indian burial sites, and native peoples are now consulted and involved in relevant projects as a matter of course.

The problems that remain involve the length of time allowed for analysis before reburial and the fate of very ancient remains, which many scholars consider to be part of the world heritage rather than directly ancestral to the people who happen to live in an area today. For example, the inclusion of 127 skeletons from Coobool Creek, dating from 14,000 years ago, in the Murray Black collection that was recently returned, distressed many archaeologists. Some of the skulls display evidence of binding in childhood to induce deformation (P. Brown, *Archs Oceania* 16, 156–167; 1981; J. Mulvaney, *Aust. Nat. Hist.* 23, 66–73; 1989), the only record of such a practice throughout Australia's 40,000 year prehistory.

It was agreed at the congress to allow sub-committees to draw up a code of ethics, but how widely their deliberations will be accepted remains to be seen. The most active group of native Americans, the 'American Indians Against Desecration' (AIAD), is a small, self-appointed body with no mandate to speak for all native Americans, whereas the World Archaeological Congress, which organized the meeting, is a small splinter-group within the community of professional archaeologists.

The possible problems ahead became apparent at the post-congress reburial of some 540 kg of miscellaneous fragments of Seminole Indians. They had been unearthed three years ago during construction work at Tampa, Florida, and the local population had asked the University of South Dakota to store them without analysis, pending arrangements for reburial. As they were never studied, one cannot even estimate how many people they represented. The reburial, led by a representative of the Seminole Tribe of Oklahoma, took place in the Pine Ridge Reservation, close to Wounded Knee, the site of the 1890 massacre of Sioux Indians. A strong media presence, however, turned the event into a political statement rather than simply a religious or symbolic act. AIAD wants the site to become a national cemetery of native Americans, where no archaeologist can ever touch the remains; but the fact that the local community and Sioux Tribal Council did not permit the reburial to take place at Wounded Knee itself, and the boycott of

the reburial by several notable native Americans involved in the cause, point to strong divisions between the different factions.

The whole emphasis of this important issue is the need for mutual respect. It is inevitable that such a sensitive problem is entwined with the political aspirations of indigenous peoples and their search for lost sovereignty: indeed, native Americans and Australian Aborigines at the congress

IMMUNOLOGY

Naked or peptide-clothed MHC?

David H. Margulies

ALTHOUGH the vertebrate immune system was never designed to defend the organism against the genetically distinct tissues presented by organ or bone marrow grafts, the cellular phenomenon of alloreactivity to histoincompatible cells is rich territory for immunological explorations. An attempt to synthesize a model of alloreactivity that is consistent not only with the important biological consequences of graft rejection but also with a detailed molecular view of the component structures was the focus of a recent conference* in Milwaukee.

Increased understanding of the molecular basis of alloreactivity is emerging from coordinated advances in our knowledge of the genetics and structure of the key molecules that mediate the alloreactive interactions between T lymphocytes and their stimulatory or target cells — namely, the T-cell receptor and the major histocompatibility complex (MHC) class I or class II molecules — together with studies on the parallel set of immune responses in antigen-specific MHC-restricted T-cell recognition.

The major discussion (controversy would be too strong a word) continues to concern the nature of the T-cell receptor's ligand in alloactions. Is this the naked MHC molecule, or is the MHC molecule complexed to particular peptides derived either from the local environment or from the target or antigen-presenting cell itself? Perhaps the most powerful recent evidence in favour of MHC-peptide complexes being the ligand came from the first X-ray crystallographic structure of an MHC class I molecule: its antigen-binding cleft contained electron-dense material not accounted for by the primary sequence of the molecule and therefore interpreted as a peptide¹.

At the meeting, several reports of cell-lineage-specific recognition by alloreactive T cells (R. Lechler, Royal Postgraduate Medical School, London; C. Sharrock, St. George's Hospital Medical School, London) were also consistent with

found that they had much in common in terms of past mistreatment and their current struggle for rights of all kinds. The treatment of human remains is, however, fundamentally a religious and moral issue; its use as a political football does little to enhance its well-deserved respectability. □

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a model in which the MHC-peptide complex is the ligand, where the peptide is derived from a cell-type specific protein. A particularly convincing demonstration of cell-lineage specific allorecognition is reported by Sherman and her colleagues² who have identified alloreactive cytotoxic T-cell clones that recognize the mouse class I H-2K^b molecule when it is expressed on mouse cells but not human cells. Addition of a crude mouse cell peptide extract to the H-2K^b-expressing human cells, however, results in their lysis by the alloreactive T cells. Thus, the allorecognition is clearly demonstrated to depend on a specific peptide antigen. Further evidence comes from *in vitro* mutagenesis studies designed to test the role in allorecognition of amino-acid residues of the MHC class I or class II molecules thought to be involved primarily in binding antigen rather than interacting with the T-cell receptor. Thus, point mutations in regions not considered accessible to the T-cell receptor can abolish allorecognition in the human class II system (G. Lombardi, University Postgraduate Medical School, London; S. Rosen-Bronson, Georgetown University School of Medicine; and R. Karr, University of Iowa College of Medicine) as well as in the human class I system (K. Hogan, Medical College of Wisconsin).

Biosynthetic variants that seem to have normal class I (P. Cresswell, Duke University) and class II molecules (T. Cotner, University of Washington) but abnormal cell-surface expression, implicate additional cellular factors in MHC molecule assembly and transport. Furthermore, a genetic locus closely linked to, but clearly distinct from, the rat MHC class I gene has significant effects on the cellular processing and T-cell antigenicity of this MHC class I molecule (J. Howard, Institute of Animal Physiology and Genetics Research, Cambridge). Taken together, these reports, along with the recently published work of Townsend *et al.*³, suggest that intracellular assembly, transport and functional cell-surface expression of MHC molecules can be controlled by other genes. It is tempting to consider a

*Molecular Mechanisms of Allorecognition, Milwaukee, Wisconsin, 12–14 September 1989.

critical role for 'antigenic' peptide in some of these cases.

Some of the strongest data to implicate antigenic peptides in allogeneic responses come from a cytotoxic T-cell line, the allo-reactivity of which is dependent not only on the presence of an allogeneic MHC molecule but also on the expression of an MHC-linked gene (D. Schendel, University of Munich). The fact that these T cells also respond to genetically identical cells that have been transformed by Epstein-Barr virus is most simply explained by the ability of the same T-cell receptor molecule to see either a peptide from Epstein-Barr virus bound to syngeneic MHC or a peptide from an MHC-linked gene bound to an allogeneic MHC molecule.

Although several experiments suggest the attractive conclusion that MHC-peptide complexes are required for allo-recognition, a few suggest that allospecificity is not always peptide dependent. One provocative analysis indicates that as many as 60 per cent of H-2L^d-specific cytotoxic T lymphocytes express the same T-cell receptor variable region family, but show no evidence of conservation of primary sequence at the V-D-J junction (J. Bluestone, University of Chicago). A favoured interpretation is that an interaction between the V-domain of the T-cell receptor and a domain of the H-2L^d class I molecule is sufficient to confer allospecificity in the case of this particular response.

The difficulty in drawing a precise picture of alloreactivity lies in the wide spectrum of interactions that T-cell receptors are equipped to form. At one extreme is the possibility that some T-cell receptors may interact only with amino-acid side chains (and perhaps even the peptide backbone) of the MHC molecule, whereas, at the other extreme, others might interact only with peptide-derived structures even though the peptide is MHC bound. These simple models are compounded by considerations of conformational changes that might be induced either in the MHC molecule on the binding of the peptide or in the peptide on the binding of the MHC molecule. The favoured model, of course, includes a little of each extreme — most T-cell receptors interact with structures determined by both the MHC molecule and the resident peptide. Thus, allorecognition increasingly becomes a set of special cases of antigen-specific MHC-restricted interactions. □

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William Martin Fairbank (1917–1989)

THE American physicist, Bill Fairbank, died on 30 September of a massive heart attack while jogging near the Stanford University campus. He was a giant among the experimental physicists of his generation, recognized for his brilliant experiments in low-temperature physics concerning liquid helium and superconductivity, and for his ingenious use of low-temperature techniques to explore many other areas of physics including elementary particles, gravitational waves and general relativity.

During the early part of his career, he and his group performed pioneering studies on the nature of ⁴He and ³He at low temperatures. They were the first to measure, in 1954, the Fermi-Dirac nature of ³He using NMR techniques at temperatures below 0.25 K. Two years later, they discovered the phase separation of ³He-⁴He mixtures at temperatures below 0.8 K, also using NMR. By exploring the phase transition of ⁴He into the superfluid state with microkelvin resolution, they showed that the heat capacity has logarithmic divergences around both sides of the critical temperature, with a discontinuity at the critical temperature.

By the late 1960s, Fairbank's group had detected the quantized angular-momentum states of rotating superfluid ⁴He and nuclear antiferromagnetism in solid ³He. The studies of superfluid ⁴He were motivated by F. London's theories of the macroscopic quantum nature of the superfluid and led naturally to studies of superconductivity, which London believed was a similar phenomenon.

Fairbank's most important contribution was the discovery, together with his graduate student B.S. Deaver, of the quantization of magnetic flux in a superconducting ring. In one extraordinary experiment, they observed quantization in multiples of $hc/2e$, demonstrating both the macroscopic quantum nature of superconductivity and the pairing of electrons in the superconducting state. Later experiments with rotating superconductors included the first observation of the London moment, a spontaneously appearing magnetic moment that is proportional to spin speed and which also arises from the macroscopic quantum nature of the superconducting state.

Later, Fairbank initiated a series of ambitious experiments which have as a common theme the application of low-temperature techniques and devices to the study of exciting questions in other areas of physics. Three of these continue as lively

and important research programmes. The first is the superconducting electron accelerator based on niobium radiofrequency cavities. The unique properties of this facility were needed in the first demonstration of the free electron laser. The second is a NASA programme to perform a fundamental test of Einstein's theory of general relativity in Earth orbit. The experiment is based on an exquisitely precise cryogenic gyroscope whose London moment is monitored. It is scheduled for a test flight in 1993 and a science mission in about 1996. The third is a search for gravitational waves, which are predicted by general relativity and are thought to be emitted during the collapse of stars into neutron stars or black holes. Detectors capable of observing a collapse anywhere within our

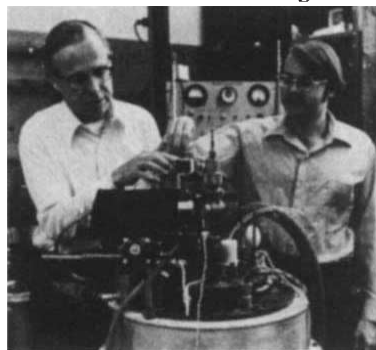
own Galaxy are in operation, and the construction of detectors capable of sensing collapses within the local Virgo cluster of galaxies are under design.

Perhaps Fairbank's most controversial experiment is the search for free fractional charge in matter (a possible indicator of free quarks), an experiment in which niobium microspheres are levitated in a mag-

netic field between capacitor plates. The motion of a sphere in an oscillating electric field is used to measure the charge on each sample. After many years of careful work, the group reported "evidence for" (in 1977) and "observation of" (in 1981) free fractional charges in matter with values of $+1/3 e$ and $-1/3 e$ modulo e . In 1986, after studying and understanding a new systematic effect related to an interaction between the magnetic levitation and the capacitor plate vertical alignment, they chose to modify the 1981 conclusion, returning to the earlier wording of "evidence for" but reiterating that the strong statistical evidence for fractional charge in both sets of earlier data remained compelling.

Over the past several years, further modifications of the apparatus have removed this systematic effect and even on the night before his death, Fairbank and his students obtained new data. Analysis of these most recent experiments is continuing. Throughout this difficult work, Fairbank's strengths were always evident. He tackled enthusiastically the most difficult fundamental experiments, remained open to new conclusions and never feared failure.

BLAS CABRERA



Fairbank (left) with G. LaRue by the fractional-charge experiment. (From *Near Zero*, Freeman, New York, 1988.)

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When protostars cease trading

A. P. Whitworth

THE final stages of star formation are accompanied by violent dynamical instabilities, as the star seeks simultaneously to accrete more material and to shed excess angular momentum. It is only when this trade in mass and angular momentum has virtually ceased that the star can be observed in its own right. By this stage the star has either accreted or blown away most of the gas and dust surrounding it. Elsewhere in this issue (*Nature* 342, 161–163; 1989) Y. Fukui and co-workers report their analysis of millimetre-wave observations of powerful molecular outflows associated with young stars from which they derive important new constraints on the duration of the early accretion phase.

Stars form from dense clumps of interstellar gas which are pulled together by self-gravity. The central regions of the clump, being the most dense, condense out as a star on a rather short timescale. Normally, the outer material has too much angular momentum to fall directly onto the star, and so in the first instance it is deposited onto a rotating circumstellar

disk. Such disks are commonly observed around young stellar objects, both in continuum emission from dust and in line emission from molecules.

Material in the disk is subject to magnetic stresses which remove angular momentum, so enabling the material to spiral inwards towards the central star. Given sufficient time this material is accreted onto the stellar equator. Much of the gravitational energy and angular momentum released by the inward-spiralling material in the disk is transported away by an outflow of matter and hydro-magnetic waves channelled along the poles of the star's rotation axis, at right angles to the disk.

Near the star this outflow often takes the form of a jet. Where this jet interacts with the ambient interstellar medium, it may excite a string of Herbig–Haro objects (small clumps of emission nebulosity whose optical-line spectrum indicates that they have been heated by a shock). Sometimes the string terminates in a bow shock which can be mapped in the infrared line

radiation of molecular hydrogen. Further out still, carbon monoxide emission at millimetre wavelengths reveals extended lobes of molecular gas receding from the star at supersonic speeds.

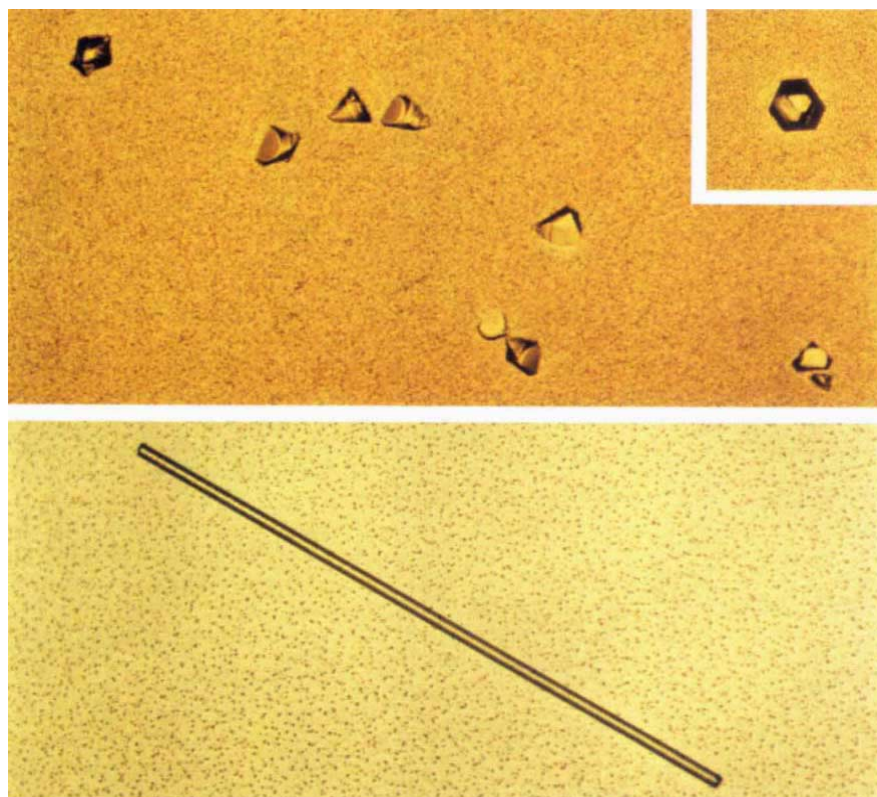
Ultimately the supply of accretable matter runs out, the accretion flow subsides, and the associated release of energy and angular momentum driving the outflow declines; the jet ceases to blow; the Herbig–Haro objects cool off; and the molecular lobes merge with the ambient interstellar medium. Parts of the residual disk may coagulate to form planets and the remainder is dispersed.

The problem is to piece together a detailed evolutionary sequence for young stellar objects, so that the different stages in the process outlined above can be distinguished observationally. The observed diversity of young stellar objects associated with disks and outflows represents a range of initial protostellar clump masses, with a range of initial angular momenta, observed at different stages in their evolution, and from different viewing angles (relative to the rotation axis). The observational data set is distorted by selection effects. The work by Fukui *et al.* is an important step towards solving this problem.

Previous investigations of outflowing

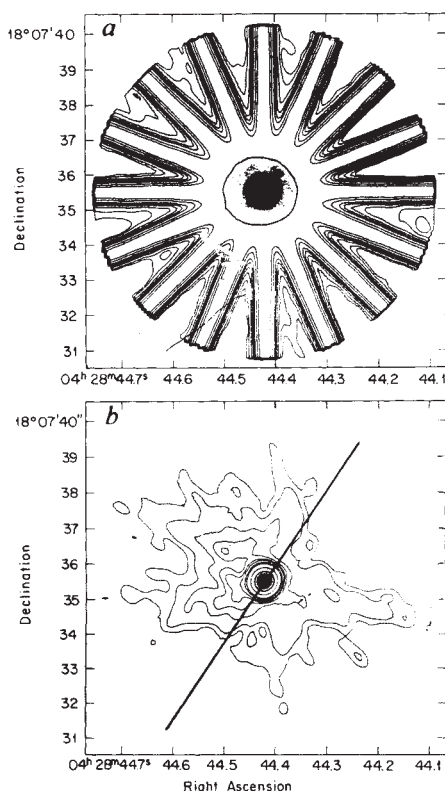
Diversity of laser snowflake crystal forms

LASER SNOW is the name given to powdery clouds first observed by A. Tam, G. Moe and W. Happer in a caesium vapour exposed to laser light (*Phys. Rev. Lett.* 35, 1630–1633; 1975). It seems that the grains consist of caesium hydride, made when caesium atoms excited by the laser radiation react with hydrogen in the ambient vapour. The particle growth is a nucleation process similar to that responsible for raindrops, which is why T. Tanaka and colleagues at Kyoto University recently joined with K. Sugiyama of the Japanese National Meteorology Research Laboratory to study their morphology (*Phys. Rev. Lett.* 63, 1390–1392; 1989). To their surprise, the crystal shapes depend exquisitely on the conditions in the vapour cell: whiskers up to 400 μm long (bottom) are produced when the temperature of the caesium–hydrogen vapour is 300 °C and the laser power is relatively low (80 milliwatts); with the same laser power, but at 250 °C, small polyhedra, typically having a hexagonal cross-section, are produced (top, inset); reducing the temperature further (to 230 °C) while raising the laser power to 300 milliwatts results in pyramidal crystallites (top, main picture). Not only does the laser promote the caesium–hydrogen reaction, but also by virtue of the photoelectric effect, it positively charges the particles. The authors argue that electrostatic repulsion prevents



the crystals from growing by the coalescence of smaller particles. Instead, they argue, individual CsH molecules condense onto the grains, a process enhanced by the 'electron harpoon' mechanism.

Caesium hydride molecules are moderately electronegative and so easily capture the crystal's photoelectrons. The resultant electrostatic pull quickly attaches new molecules onto the crystal surface. □



Tomographic imaging of HL Tau. A 'dirty' map of HL Tau (a) is produced by superposing eight speckle images, each generated by analysing the 2- μ m intensity observed through a slit during a series of rapid scans across the star. Each speckle image corresponds to a different orientation of the slit. Speckle analysis enables astronomers to compensate for the blurring due to turbulence in the Earth's atmosphere (which limits the resolution of conventional long-exposure images). The 'clean' 2- μ m map (b) is obtained by deconvolving the dirty map with the equivalent dirty map of a point source. The diagonal line indicates the plane of the disk around HL Tau. (From Beckwith *et al. Astrophys. J.* **343**, 393; 1989.)

molecular lobes have selected sources on the basis of optical evidence of young stellar activity. To by-pass this selection effect, Fukui *et al.* have made a complete survey of carbon monoxide emission (in the $J = 1 \rightarrow 0$ rotational transition at 2.6 mm) from the dark cloud L1641.

The authors have detected five new outflow sources, and have confirmed a sixth, on the basis of broad Doppler wings on the spectral line profile observed at six positions in the cloud. These broad wings are taken to represent emission from outflowing gas with a large systematic velocity relative to the bulk of the carbon monoxide (rather than gas with a higher temperature).

These six sources represent all the bipolar outflow sources in L1641 whose mechanical energy output exceeds 10^{-3} solar luminosities. The mass outflow rate in the molecular lobes is typically 10^{-5} solar masses ($M_{\odot}$) a year. Most of this mass comprises swept-up ambient interstellar matter rather than material derived

directly from the newly formed star. All six outflows are coincident with IRAS sources detected by the Infrared Astronomical Satellite. By comparison with the IRAS sources associated with T Tauri stars in the same cloud, the outflow IRAS sources are cooler, more luminous and more massive than the T Tauri IRAS sources. (T Tauri stars are the most commonly observed category of young low-mass star.)

In both cases, the radiation detected by IRAS is presumed to be thermal emission from hot dust in a circumstellar disk. Fukui *et al.* conclude that the outflow sources represent an earlier phase of evolution than do the T Tauri sources, characterized by a higher rate of accretion and hence a larger, cooler disk, and a higher luminosity. They estimate that if the excess luminosity is generated by release of gravitational energy in the accretion flow, then the required accretion rate is $10^{-5} M_{\odot} \text{ yr}^{-1}$.

By comparing the relative numbers of outflow sources and T Tauri stars, Fukui *et al.* conclude that the phase of rapid accretion represented by the outflow sources lasts about 10^5 yr; the subsequent T Tauri phase, characterized by more modest accretion rates and infrared luminosities, lasts for $2-3 \times 10^8$ yr. This calculation presupposes that star formation in this locality proceeds more or less continuously.

If this conclusion is correct, the question arises: how rapidly does accretion subside in the T Tauri phase? Recent observations by S. V. W. Beckwith *et al.* (*Astrophys. J.* **343**, 393-399; 1989) suggest that the disk around the T Tauri star HL Tauri (in the constellation Taurus) is still accreting mat-

ter at a rate of $3 \times 10^{-6} M_{\odot} \text{ yr}^{-1}$. Provided the inflow is in a quasi-steady state, this is also the rate of accretion onto the central star.

Beckwith *et al.* have used a novel speckle-interferometric technique, which they call tomographic imaging (see figure), to reconstruct an image of the near infrared emission (at 2 μ m) from HL Tau. From the form of this image they infer that the disk around HL Tau is seen roughly edge-on, and that the 2 μ m radiation originates in the obscured central star and is scattered towards the observer off diffuse dust well above (and below) the plane of the disk.

The authors argue that this scattering dust must be involved in matter infalling onto the disk at a rate of $3 \times 10^{-6} M_{\odot} \text{ yr}^{-1}$. They reject the alternative explanation that the scattering dust is involved in outflow, having been entrained at the edge of a stellar wind cavity where the wind impinges on the ambient medium, because this explanation requires an unacceptably high mass-loss rate and wind-speed. This calculation, however, presupposes a steady-state of mass loss and accretion; the shape of the 2- μ m image is remarkably suggestive of the limb-brightened image that would be produced by a stellar wind cavity.

Impressive as these observations are, their full bearing on the process of star formation will probably only become clear when the tomographic technique has been applied to a statistically significant sample of sources. $\square$

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PROTEIN SYNTHESIS

Elongation remodelled

Peter B. Moore

TWENTY-FIVE years ago James Watson proposed¹ a two-site model for the ribosomal phase of protein synthesis that made sense of what was known at the time, and which is still widely accepted, as the reader may confirm by examining any biochemistry textbook of recent vintage. The message implicit in the paper by Danesh Moazed and Harry Noller of the University of California, Santa Cruz, that appears on page 142 of this issue², is that the model, as originally formulated, is not an adequate description of the process of peptide chain elongation in the ribosome; it is time to revise the textbooks.

According to the two-site model, a messenger RNA-programmed ribosome in the initial state of the elongation cycle has a peptidyl transfer RNA molecule bound to its P(eptide) site. In the first step of the cycle, an aminoacyl tRNA, which has an anticodon complementary to the

mRNA triplet presented by the ribosome, binds to the A(minoacyl) site of the complex. Binding is catalysed by the elongation factor, EF-Tu, and leads to the hydrolysis of a GTP molecule. The peptide on the P site tRNA is then transferred to the amino group of the amino acid on the A site tRNA by an activity known as peptidyl transferase, which is intrinsic to the ribosome. This transfer creates an A-site-bound tRNA carrying a peptide that is one residue longer than it was at the beginning of the cycle. In the last step of the cycle, translocation, the new peptidyl tRNA is moved from the A site to the P site, displacing the now discharged P site tRNA, and the messenger is advanced by one triplet. Translocation is catalysed by EF-G, is driven by GTP cleavage, and restores the system to its original state.

Both ribosomal subunits contribute to the A and P sites; anticodon-mRNA

interactions are small subunit functions, whereas peptide transfer, which involves the aminoacyl end of tRNA, is localized on the large subunit. The two-site model postulates that tRNAs move from A site to P site on both subunits during translocation, under the influence of EF-G; the tRNAs are either entirely in the A site or entirely in the P site.

The new findings of Moazed and Noller indicate that whereas the aminoacyl end of a site-bound tRNA moves to the P site on the 50S subunit at the time of peptide transfer (before translocation), movement of its anticodon end from A to P on the 30S subunit takes place during translocation and requires the action of EF-G. Thus movement from A to P is achieved in two steps, not one, and an intermediate state exists in which the anticodon end of the tRNA is still in the A site on the small subunit while the aminoacyl end occupies the P site of the large subunit.

Noller's group uses primer extension techniques to examine the chemical reactivity of ribosomal RNAs in the ribosomes of *Escherichia coli*. Their previous work identified bases in both 16S and 23S rRNA whose extent of reaction with small reagents is reduced when tRNAs bind to specific ribosomal sites ('protections'). The current paper, which deals with the changes in site occupancy that occur during the elongation cycle, uses these protections as structural indicators.

In addition to identifying large and small ribosomal subunit bases specific to the classic A and P sites, Moazed and Noller's protections identify residues associated with a third tRNA-binding site, the E(xit) site, through which discharged tRNAs pass as they leave the ribosome. The E site is localized mainly on the 50S subunit, and tRNAs appear to interact with it through the CCA sequence at their 3'-termini. That there might be such a site was suggested many years ago³, but only in recent years has strong evidence appeared proving its existence (see ref. 4).

Moazed and Noller formulate an attractive model for the elongation cycle based on their observations. It postulates a two-step movement of tRNAs from the P site to the E site that parallels the movement from A to P. The aminoacyl end of a P-site-bound tRNA moves to the E site at the time of peptide-bond formation; the anticodon end of the tRNA leaves the P site on the 30S subunit only during translocation. The model suggests that tRNAs interact with the ribosome mainly at their ends, which may explain their L-shaped tertiary structures. The model's steps ensure that tRNAs are bound to the ribosome at one end or the other at all times, accounting for the processive character of protein synthesis, which the old two-site model does not. As the authors point out, the two-step transfer of tRNAs from A site to P site, and from P

site to E site could result from reciprocating motions of the two subunits. This is an ancient notion in the protein synthesis field^{5,6}, and it rationalizes the two-subunit organization of the ribosome, which is a universal feature of ribosome structure.

In interpreting their data, Moazed and Noller assume that the A, P and E protections observed result from tRNA interactions with distinct sites on the surface of the ribosome. Read this way, their data imply that tRNAs progress from one site to the next during elongation in the way their model proposes. The connection between protection and structurally distinct sites, however, is not tested in these experiments. Changes in the accessibility of ribosomal residues in response to alterations in physiological state that are not associated with tRNA movements are not excluded. For this reason, the data must be seen as being consistent with the model, but not as proving it conclusively.

It remains to be seen whether this new model for elongation will enjoy the longevity of its distinguished predecessor. It is certain to be subject to searching experi-

ASTRONOMY

New pulsar confounds models

Andrew Lyne

THE discovery of a millisecond pulsar (rapidly spinning neutron star) in a globular cluster, two years ago, provided strong circumstantial evidence that old pulsars can be spun up to millisecond periods by mass accretion in low-mass X-ray binaries. Since then, a total of ten short-period pulsars have been found in globular clusters; moreover, most millisecond pulsars are seen to be in binary systems, lending further support to the theory. On page 158 of the issue¹, Ables *et al.* describe the details of one of the ten, PSR0021-72A, a remarkable object in the globular cluster 47 Tucanae that flies in the face of expectations. If correctly interpreted, it may provide new high-precision tests of relativity and gravitation.

This pulsar has a rotation period of 4.5 milliseconds and shows a phase modulation of the arrival times with an amplitude of about 2 milliseconds and a period of 32 minutes. The most straightforward interpretation of this modulation, and the one adopted by Ables *et al.*, is that the pulsar is a member of a binary system with an extremely small mass function of 3×10^{-8} solar masses ($M_{\odot}$; the mass function, derived from the observations, can be used to infer properties of the binary). The measured advance of periastron (point of closest approach of the two stars) and a periodic variation in pulse arrival time are interpreted as being due to general relativistic effects. The derived masses of the pulsar and the companion

provide values of $1.4 M_{\odot}$ and $0.8 M_{\odot}$, quite reasonable values for a neutron star and a white dwarf, respectively. But there are a number of strange and unexpected consequences of this interpretation.

The first of these is that the orbit has a very special orientation, having an inclination of only 0.38° to the plane of the sky — it is nearly face on to the Earth. For a random distribution of orientations, only one in about 45,000 binary orbits would have such a small inclination. Because only 13 binary pulsar systems are known, this is clearly a most improbable occurrence.

The second unexpected result, given that the inclination is so small, is that we see any pulses at all. In the spin-up model, angular momentum is transferred from the orbital motion to the spinning neutron star, so that the rotation axis of the pulsar will end up normal to the plane of the orbit, pointed towards the Earth. This is the one orientation in which we would not expect to observe any pulses, certainly not the fairly narrow ones recorded.

The final surprise is that the orbit is highly eccentric — its departure from a circular orbit (eccentricity) is 0.32. This is certainly not expected after spin-up in an accretion process, which should render any orbit circular. It immediately suggests that the pulsar was not spun up in the system we see today. Ables *et al.* and Wijers² suggest that the present system is the result of the collision of another star with

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the binary system containing the spun-up pulsar. This encounter eventually left the pulsar in a deformed orbit either with the new star or with the star originally responsible for the spin-up.

This could certainly account for both the eccentricity and misalignment of the pulsar spin and orbital angular momenta. Such collisions are probably quite rare even in dense clusters such as 47 Tucanae; calculations suggest there would be only one collision in the lifetime of the cluster (10^6 yr). In this case it must have happened fairly recently, within the past 10^7 yr or so, otherwise gravitational radiation would have reduced the eccentricity below the presently observed value. Moreover, within about 10^6 yr, gravitational radiation will cause the system to lose energy and the pulsar and companion to coalesce. So the system as we see it now has a very short lifetime compared with the probable lifetime of the pulsar, which could be a hundred times greater. Again, we are seeing the system in a special situation, very near the end of its life.

The statistical implication of this is that there are a great many similar systems. Several authors³⁻⁵ are concerned that the large number of millisecond pulsars is something of an embarrassment to the spin-up theory, as there do not seem to be enough low-mass X-ray binaries (LMXBs) to give a sufficient rate of production of millisecond pulsars. Ables and co-workers' interpretation of the discovery clearly exacerbates the problem⁶. The production rate is determined by the long lifetime ascribed to the LMXB accretion phase. It is suggested⁶ that the problem may be resolved if the length of this LMXB phase were curtailed within about 10^7 yr by a mechanism proposed by Ruderman *et al.*⁷, in which radiation pressure from the spun-up pulsar may quench the accretion process.

Because of the three rather awkward implications presented by the interpretation of Ables *et al.*, it is worth considering other possibilities not entertained by them. The first is that the very small mass-function should be taken at face value and

that a $1.4-M_{\odot}$ neutron star is in orbit with a very light body ($4 \times 10^{-3} M_{\odot}$) and with a reasonable inclination of the plane of the orbit to the sky. This Jupiter-like mass would be orbiting the neutron star at a radius of perhaps 100,000 km. This system would be rather like that in which PSR1957+20 has nearly ablated its companion by radiation. It may be significant that the three pulsars with periods shorter than PSR0021-72A are either solitary, presumably having already evaporated their companions, or are in the process of becoming so. The observed advance of periastron in this case would be due to classical effects resulting from the unknown, extended nature of the companion. The apparent finite eccentricity of the orbit is the main unexplained aspect of this interpretation, although it has been suggested⁷ that this could arise during the ablation process.

More speculatively, perhaps the 2-ms phase modulation can be explained in some other way: for example, the rotation axis of a distorted neutron star could freely precess; or an exchange of angular momentum between the crust and solid

core of the neutron star could take place.

In the absence of any such detailed theory, one of the two binary interpretations would seem to be the most satisfactory. That of Ables *et al.* indicates a most exciting astrophysical laboratory with observable general relativistic effects that are an order of magnitude greater than those seen in the Hulse-Taylor system, which contains PSR1913+16. However, it seems unwise to ignore the sheer improbability of this configuration, and a more likely, if less attractive, classical solution should be sought. $\square$

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EVOLUTION

Pruning the tree of life

Michael J. Benton

THE enormous diversity of life is the result of many evolutionary radiations over billions of years. Key questions about the overall diversification of life and especially individual adaptive radiations were tackled at a recent meeting*.

The best-known example of radiation, that of the placental mammals after the extinction of the dinosaurs 66 million years ago, shows many typical features which can be divided into three stages: rapid initial proliferation of adaptive types, as the mammals radiated to fill the ecological space just vacated by the dinosaurs; early extinctions of several lineages as the ecological space became filled and species competed with each other; and

stabilization of the group and evolutionary fine tuning with no further originations of major lines. The first two stages, giving rise to the approximately 20 orders of modern placental mammals, took about 10 million years.

Adaptive radiations of this sort have generally been explained in the past by one of two broad models — the opportunistic model and the key-adaptation model — or a combination of both (S.M. Stanley, Johns Hopkins University). Some interpret the radiation of placental mammals as pure opportunism — the radiating group might just as well have been the frogs, lizards or snails, all of which were present, but the mammals got going first. Others stress a 'key adaptation' that made it inevitable that the mammals would actually succeed: efficient teeth or limbs, intelligence, warm-bloodedness or parental care could be such adaptations.

The key-adaptation model, cited to explain evolutionary models in many groups, seems particularly convincing when applied to marine molluscs. P.W. Skelton (Open University) argued that the present diversity of bivalves (cockles, mussels, oysters and their kin) can be traced to the evolution of key adaptations to avoid predation. The appearance of adaptations such as the ability to burrow

the Darwinian doctrine, and the application of it and its subordinate conceptions in a variety of fields of investigation.

The nucleus of the protoplasmic cell — which twenty years ago had fallen from the high position of importance accorded to it by Schwann — has, through the researches of Bütschli, Flemming, and Van Beneden, been reinstated, and is now shown to be the seat of all-important activities in connection with cell-division and the fertilization of the egg.

The British Government has shown a decided inclination to extend subsidies to scientific research: the grant to the Royal Society has been increased from £1000 to £4000 a year; and subsidies have been voted to the Marine Laboratory, the Committee on Solar Physics and the University Colleges.

From *Nature* **XLI**, 1; 7 November 1889.



100 years ago

A REMINDER that today is the twentieth anniversary of the first issue of *Nature*, will not, perhaps, be without interest to our readers. A formal history of science for the last twenty years would be a formidable task, but it is already possible to discern what will probably appear to posterity to be the most salient characteristics of the last two decades.

In the biological sciences, progress has consisted chiefly in the firm establishment of

* Major evolutionary radiations, Durham, 6-8 September, 1989. To be published in 1990 as *Systematics Association Special Volume 38* (Oxford University Press).

into the sea bed, the ability to become cemented to hard substrates, or the ability to escape by swimming (scallops), was generally followed by a rapid proliferation of the group.

J.D. Taylor (British Museum (Natural History)) similarly showed how the success of neogastropods (20,000 species) can be traced back to their radiation in the Cretaceous, some 100 million years ago, when they began to evolve a fiendish array of predatory adaptations. Some can bore holes into hard-bodied prey through which they suck their victims' flesh; others can catch prey with a long snake-like proboscis; some swallow their prey whole by powerful suction; others have complex venom glands and even modified hollow 'teeth' that are shot into their prey like hypodermic poison darts. Unlike many of these predatory adaptations, hole-boring predation can be tracked back in the fossil record. Fossil shells with holes that were bored by particular gastropods became more common during the Cretaceous. This occurred in parallel with the radiation of the hole-borers, thus providing cogent evidence for the radiation and effectiveness of at least this adaptation.

Other contributors found less evidence that key adaptations could explain the radiations of their chosen groups of organisms. M.B. Hart (Plymouth Polytechnic) emphasized the role of repeated extinction events and opportunistic radiation in the fossil record of planktonic foraminifera. After an extinction event, the survivors, which seem to have been mainly surface-living forms, radiated to occupy deeper positions in the water column. Extinctions over the past 250 million years have also triggered radiations of echinoderms (M.J. Simms, Trinity College, Dublin) and terrestrial tetrapods (A.R. Milner, Birkbeck College, London; my own work). By contrast, evolutionary radiations of colonial animals involved major structural changes in the colony form that are best explained by heterochronic change. Evidence was presented for the corals (B.R. Rosen, British Museum and J.M. Pandolfi, Australian Institute of Marine Science), bryozoans (P.D. Taylor, British Museum; G.P. Larwood, University of Durham), and for the extinct, largely planktonic colonial graptolites (C.E. Mitchell, State University of New York, Buffalo).

Several contributors addressed the so-called null or neutral model, which states that species may diversify by chance alone, and not for any particular biological or physical reason. Although the issue was considered in biological and statistical terms, the consensus was very much against this model.

Radiations at higher levels than the individual adaptive radiation may also yield general patterns and models of

evolutionary interest. The diversification of the tetrapods, for example, is largely the result of the conquest of new 'adaptive space' and the subdivision of niches, or increasing specialization of species and families. There does not seem to have been any in-built progressive 'tendency' in their evolution. D. Jablonski (University of Chicago) and D.J. Bottjer (University of Southern California, Los Angeles) note that marine invertebrates have repeatedly shown environmental patterns of radiation. Most of the main groups arose in shallow waters and then migrated over tens of millions of years into deeper seas. New groups then replaced them in the near-shore waters. This remarkable 'conveyor belt' is unexpected and may have parallels elsewhere. Skelton and co-authors find evidence that most of the main bivalve groups arose at high latitudes and subsequently migrated towards equatorial seas; Jablonski and Bottjer, however, find that most other marine groups arose at low latitudes and migrated north and south. Similarly, P.R. Crane and S. Lidgard (Field Museum of Natural History, Chicago) report an equatorial origin for flowering plants, with a subsequent migration to temperate latitudes.

A potential problem with studies of evolutionary radiations is the incompleteness of the fossil record. This may be a serious problem in studies of the well known Early Cambrian radiation, some 570 million years ago, when hard parts first appeared in the fossil record, and molluscs, arthropods, echinoderms and others apparently radiated very rapidly. It is not known whether this seemingly rapid radiation is preceded by a significant gap in the fossil record (S. Conway Morris, University of Cambridge). Most parts of the fossil record are, however, complete enough for valid macroevolutionary studies, with many hundreds of fossil deposits showing exceptional preservation. These have provided information on soft-bodied organisms, as well as hard-bodied organisms with delicate structures. Together, they enable palaeontologists to interpret with confidence those intervening parts of the fossil record showing poorer preservation.

The other key problem in studying radiations is establishing the precise geometry of such patterns, the shape of the tree of the history of life. Most of the 21 studies presented at the meeting were based on new cladistic analyses, a rigorous technique for assessing patterns of lineage splitting. Cladistic techniques are becoming the norm for macroevolutionary studies and promise increasing success for the palaeobiological study of evolutionary radiations. □

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Floating on air

THE hovercraft supports itself on a cushion of pressurized air, maintained between its underside and the ground. But lacking sides, the cushion leaks absurdly freely. Air has to be pumped down into it at an enormous rate to sustain the pressure. Daedalus is now designing a novel leak-proof hovercraft. It pumps air inwards into its cushion from all round its periphery, exactly opposing the outward leakage.

The basic principle is that oxygen, and therefore air, is paramagnetic. So it can be entrained and pumped by a moving magnetic field. The periphery of the DREADCO hovercraft is studded with linear magnetic motors, whose field pattern runs radially inwards at high speed. These motors pump air in under the craft until sufficient pressure has built up to oppose further inward flow. Acting on weakly paramagnetic air, even the most powerful linear motors can only pressurize the central cushion by a tiny fraction of an atmosphere — but over the whole bottom area of the craft this should be enough to float several tons.

This novel vehicle has two great advantages. First, once established, its supporting air cushion will be quite static. With no fans or air draughts, the magnetic hovercraft will be utterly silent. It will float above the ground as enigmatically as a flying saucer. Second, the magnetic pumps merely serve to maintain a static pressure-gradient across the peripheral air of the cushion. They do no work of compression and with luck will consume very little energy. The craft should be very efficient.

As with all hovercraft, propulsion poses a problem. Bleeding off cushion air for jet propulsion would sabotage its lift; biasing the linear motors to drive the craft along a metallic track would tie it down like a train to a railway. Daedalus hopes to build a novel jet engine on the same principle as his magnetic air-pump: a long tubular linear motor, sucking air in at one end and blowing it out the other. Even modest thrust from such a quiet and simple engine should give his frictionless vehicle a useful turn of speed. And two parallel engines could be biased against each other to steer it.

The magnetic hovercraft should be very popular with environmentalists and nature lovers. Alone among heavier-than-air vehicles, it will produce no downwash. It will travel quietly over crops and meadows and swamps and tidelands, without alarming the wild life or flattening the vegetation. In towns, however, its stray field may be something of a liability. It will be greatly perturbed by iron manhole covers and tram tracks, and may ingest beer cans and scrap metal. Daedalus plans to give the craft magnetic sensors which momentarily switch off any linear motor that encounters such embarrassing challenges. David Jones

Carrier screening in CF

SIR—P.N. Goodfellow (*Nature* **341**, 102–103; 1989) disagrees with Kerem *et al.*, who suggest (*Science* **245**, 1073–1080; 1989) the postponement of carrier testing for cystic fibrosis (CF) until all the mutations have been identified. Goodfellow states that carrier testing is worthwhile now. A serious problem associated with immediate screening, however, is that it will reveal many couples in which one member carries the CF mutation, whereas for the other carriership cannot be excluded. Such couples are at significantly increased risk of having a child with cystic fibrosis, without the option of prenatal diagnosis or artificial insemination by donor. For a population in which 1 person in 25 is a carrier and assuming that two-thirds of the mutations are detectable (*Science* **245**, 1066–1073; 1989), 5.2 per cent of couples would have to be told that their risk has changed from 1 in 2,500 to 1 in 300. It is questionable whether this would be counterbalanced by the advantage of detecting 4 out of 9 couples with a 1 in 4 risk.

In general, if a is the proportion of mutations detectable, and q the preva-

lence of carriers in the population, the prevalences of different mating types and their risk of having an affected child are shown in the table.

The larger the proportion of detectable mutations, the less is the increase in risk

Type of mating*	Prevalence in population	Risk of having affected child	Options available
$m \times m$	$a^2 q^2$	1/4	yes
$m \times n$	$2aq(1-aq)$	$(1/4)(1-a)q$	no
$n \times n$	$(1-aq)^2$	$(1/4)(1-a)^2 q^2$	no

*m, person in which the mutation is demonstrated; n, person in which the mutation is not demonstrated

for $m \times n$ couples. Only when the proportion of undetectable mutations ($1-a$) is less than the carrier frequency (q), will the after-screening risk for $m \times n$ couples of having an affected child become less than the before-screening risk. This means that with CF at a carrier frequency of 1 in 25, screening should be postponed until 96 per cent of the mutations can be detected.

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Angiosperm origins

SIR—Martin *et al.*¹ attempt to date the origin of angiosperms by analysing sequences of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in nine angiosperm species, assuming an approximately constant (clock-like) rate of nucleotide substitution calibrated largely by estimated divergence times in other eukaryotes. They conclude that angiosperms first split into monocotyledons and dicotyledons in the Carboniferous, then into Magnoliidae and four 'higher' dicot subclasses in the Permian. They use these results to argue that angiosperms diversified much earlier than the first generally accepted angiosperm fossils in the Early Cretaceous. But they underestimate the strength of the relevant fossil evidence and neglect crucial methodological issues.

Cladistic analyses indicate that angiosperms are related to Bennettitales and Gnetales, implying that the angiosperm line was distinct by the Late Triassic^{2,3}. But despite equivocal evidence of Triassic angiosperms^{4,5}, fossil data clearly indicate that their main radiation did not occur until the Cretaceous. This view is not based solely on the absence of angiosperms in earlier rocks. The earliest unequivocal angiosperm pollen grains

(Hauterivian-Barremian) are of the monosulcate type restricted to magnoliids and monocots — they are not 'highly diversified'¹. Similar monosulcate angiosperm pollen may have escaped recognition before the Cretaceous. But the four higher dicot subclasses all have multi-aperturate pollen ultimately derived from the distinctive tricolpate type, which appears near the Barremian-Aptian boundary and soon becomes ubiquitous. This appearance is difficult to ascribe to independent origin of tricolpate pollen in several long-distinct lines because the higher dicots appear to form a monophyletic group⁶. Subsequently, angiosperm pollen and macrofossils (which now include many reproductive structures) become steadily more abundant and diverse, but even by the early Cenomanian only magnoliids and relatively primitive hamamelids and rosids have been recognized. We submit that this orderly sequence, which is based on rich pollen floras from both hemispheres and agrees with relationships derived from cladistic analyses, casts serious doubt on the molecular clock assumption¹.

The study¹ is also weakened by failure to include other seed plant groups ('gymnosperms'). The implication that monocots and dicots are sister groups^{1,7} is based on midpoint rooting, which is invalid if rates of evolution are unequal, unlike rooting with outgroups⁸. It should be noted that

their result is consistent with trees with angiosperms rooted among Nymphaeales, Piperales and monocots⁹, which are almost as parsimonious morphologically as trees with angiosperms rooted near Magnoliales⁶. Martin *et al.* also suggest that their data may favour a polyphyletic origin of angiosperms. But besides ignoring strong evidence that angiosperms are monophyletic^{2,3,9}, this view is unwarranted without inclusion of gymnosperm groups. Addition of gymnosperms could also shed light on age estimates. If angiosperms are separated from gymnosperms by a branch two-thirds as long as branches within angiosperms, as they are with ribosomal RNA data (ref. 9; E. A. Zimmer, personal communication), the inferred differentiation of seed plants would predate the first evidence of land plants (Ordovician). We hope such a result would be taken as further evidence against a molecular clock^{10,11}.

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SIR—Our recent analysis¹² of extensive chloroplast and nuclear DNA sequence data firmly supports Martin *et al.*'s evidence for a pre-Cretaceous origin of angiosperms. But our estimate of the monocot–dicot divergence at 200 million years (Myr) ago (with an uncertainty of about 40 Myr) is much younger than their estimate of 319 ± 33 Myr. (Note that the monocot–dicot divergence date represents a minimum age for the angiosperms, if one assumes that angiosperms are monophyletic.) Our estimate appears to be more reliable as it is based on much more sequence data.

The origin of angiosperms at 200 Myr ago seems to be more reasonable. As pointed out by Cleal⁷, Martin *et al.*'s estimate is ~150 Myr before the appearance of indisputable angiosperms in the fossil record. Although angiosperms probably did arise in 'upland' areas, it seems unlikely that they took so long to occupy the lowlands. Another difficulty is that because the earliest land plant fossils are only about 420 Myr old, Martin *et al.*'s estimate would imply that the lineages leading to bryophytes, pteridophytes,

gymnosperms, monocots and dicots all appeared within the first 100 Myr of land plant evolution, or these fossils do not represent the common ancestor of all the preceding lineages. In conclusion, our estimate is more compatible with the fossil record and fits well with Cleal's view.

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MARTIN, GIERL AND SAEDLER REPLY—Molecular phylogenies¹³ and estimates of angiosperm age¹⁴ were derived many years ago through protein sequencing of plant mitochondrial cytochrome *c* in the laboratory of Don Boulter. The ages suggested in these pioneering studies vastly exceeded the lower Cretaceous and were interpreted by Van Valen¹⁵ as evidence for a higher rate of substitution within angiosperms. Implicit therein is the assumption that the angiosperm fossil record would permit calculation of rate for comparison. This, is the salient issue in our contribution¹; *Sanmiguelia* is a case in point.

Crane *et al.* are quite critical of our arguments concerning angiosperm age. Is the fossil record of angiosperm history sufficiently complete to preclude the search for further specimens? We agree that fossil pollen represents an excellent monitor of total angiosperm diversity through time, although we do not recognize a compelling rebuttal of Axelrod's arguments. Seeds, required to conquer drier habitats, clearly evolved very early in land-plant evolution^{16,17}. Regarding equality of rate, we note that relative rate tests performed both with yeast and bryophyte GAPDH strongly support our arguments. Are relative rate tests applicable to phenotypic evolution? Parsimony cladistics³ indicate Triassic angiosperm origins, yet accept 33 changes of character state (including 10 reversals) to derive *Gnetum* from a putative progenitor which required only three changes to fulfil angiospermous criteria for the characters examined; is *Gnetum* thus presumed to have evolved 10 times faster? The polyphyly debate for carpel closure can only be resolved with molecular methods if representatives from the distinct gymnospermous progenitor clades in question persist in extant floras.

We concede that Wolfe *et al.*'s careful estimate¹² for the time of monocot–dicot divergence is based on a 20-fold-greater number of total nucleotide sites than our own¹. Yet maximizing the number of sites for comparison reduces the number of species from which these have been

sequenced. In the absence of suitable outgroups and for clades represented by a single species, constancy of rate within the clade was, by necessity¹², assumed.

A true consensus has yet to be reached with regard to the nature of putative angiosperm ancestors¹⁸. Until crucial 'missing links'¹⁶ for angiosperms are found, reconstruction of flowering plant phylogeny could prove a difficult problem for some time to come.

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Cysteine or serine proteinase?

SIR—We have noticed an apparent discrepancy in the sequence alignment published in a recent scientific correspondence by Higgins *et al.*¹ in reporting the possible identification of a new cysteine proteinase in *Plasmodium falciparum*. The cysteine at amino-acid position 22 of papain was incorrectly labelled as the active site cysteine. This cysteine has been shown to form an essential disulphide bridge in papain, whereas Cys 25 of papain has been shown by crystallographic and chemical studies to be the catalytic cysteine^{2,3}. Cys 25 forms an ion pair with the imidazole of His 159, with Asn 175 completing the essential catalytic triad of papain. These residues are conserved in cysteine proteinases of highly divergent organisms.

It is interesting that the amino-acid residue which is in the correct catalytic site of the *P. falciparum* protein is a serine. All known serine proteinases contain a catalytic triad (composed of histidine, aspartic acid and the catalytic serine) that has convergent active-site geometry with the cysteine proteinases. The presence of a serine at the catalytic site of the *P. falciparum* antigen indicates that this protein is actually a serine proteinase with a cysteine proteinase conformation, a structure that is similar to a class of cysteine trypsin-like proteinases found in viruses⁴.

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SIR—Higgins *et al.*¹ showed that the 111K

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antigen of *Plasmodium falciparum* has significant similarity to cysteine proteinases. We wish to point out that the cysteine labelled as the putative active-site residue is in fact involved in a disulphide bridge in papain, actinidin and probably other cysteine proteinases⁵. The gene sequence for the 111K antigen predicts a serine at the active-site position (residue 588; see refs 6 and 7). Although it has yet to be confirmed that the protein itself contains an active-site serine, as post-transcriptional modification could result in the generation of a cysteine⁸, the presence of a serine would have functional and evolutionary implications. The serine could have arisen from a cysteine by a single base mutation (TGC → TCC), with a subsequent change to the present serine codon, TCA. This would support the suggestion that proteinases with serine at the active site may have evolved from cysteine proteinases⁹. Could this malarial protein represent an intermediate between these two classes of proteinase?

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Crystal Enigma

SIR—In finding the atomic arrangements in crystals the amplitudes of the Fourier terms observed by X-ray diffraction must be combined with the correct phases to give a picture of the structure. Since this was first done by W.H. and W.L. Bragg in 1929, it has been recognized that centro-symmetric crystal structures, where the phases of the sine waves are either 0 or π , are far easier to handle than non-centro-symmetric structures, where general phase angles have to be found (nowadays by the methods of Karle and Hauptmann or by the introduction of heavy atoms as phase markers).

Protein crystals, containing as they do, L-amino acids, are always non-centro-symmetrical. But the total synthesis of protein molecules from their constituent amino acids is now possible so that, starting from D-amino acids and L-amino acids separately, it would be possible to make both D- and L-forms of the same protein of known sequence but unknown confor-

mation. Co-crystallisation of equal numbers of D- and L-molecules might produce crystals of a racemate which could be centro-symmetrical with a consequent simplification in the process of solution.

Note that the outcome of the Second World War turned on this issue, as the German Enigma coding machine was made centro-symmetrical for administrative convenience (coding and decoding procedures were then the same, and in a setting where typing in A gave B, then typing in B would return A). This weakness allowed M. Rejewski, J. Różycki and H. Zygalski, and later others at Bletchley Park, an entry to the decipherment of Enigma, which perhaps ultimately led to military defeat of the Axis. Probably no crystallographers were involved on either side.

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Pre-Quaternary Milankovitch frequencies

SIR—According to the Milankovitch theory^{1,2} external climatic forcing results from changes in the orbital parameters of the Earth's path around the Sun which affect the amount of solar radiation received at the top of the atmosphere. The main periods during the past 5 Myr of the three most important parameters, recalculated in ref. 3 are: eccentricity (a measure of the shape of Earth's orbit around the Sun) — 413, 95 and 123 kyr;

obliquity (the tilt of the Equator on Earth's elliptical orbit around the Sun) — 41 and 54 kyr; climatic precession (a measure of the Earth–Sun distance at the summer solstice) — 23 and 19 kyr. Evidence of these periods has been found in sedimentary data recording the Quaternary climate^{4,5}.

Recent results from sediment records have shown that the pre-Quaternary climate was also dominated by components with periods ranging between 20 and 100 kyr (ref. 6). To relate this kind of periodicity to astronomical forcing, the frequencies of the latter must be calculated accurately. These can be obtained from a theory that describes the gravitational effects of the Sun, the Moon and the planets on the Earth's orbit. They include the effect of the Earth–Moon distance which is known to have changed during geological times and could affect the periods of astronomical forcing. Therefore, we must understand how the variations of the lunar orbit, and the related variations of the Earth's rotation and shape, can influence the Earth's orbital frequencies⁷. The complexity of the problem is increased by the interrelations between these parameters⁸: for example, the Earth's rotation rate is related to the Earth–Moon distance through tidal friction, and it influences the shape of the Earth through the centripetal force.

Precession and obliquity can be expressed as quasi-periodic

functions of time³ where the periods involved and the associated amplitudes are a function of the 'constant' of precession (this is not the case for the eccentricity). This 'constant' in turn depends on the three parameters just mentioned. Here we use Earth's rotation rate deduced from fossil corals or observations of ancient eclipses⁹. The associated variations of the other two parameters (the Earth–Moon distance and the Earth's shape) are also available in the literature^{10–12}.

With this whole set of data and appropriate models we can calculate the long-term variations of the main astro-climatic periods over the past 500 Myr (ref. 7). The figure shows that these periods have always been smaller than today's, their shortening becoming rather significant for epochs older than 100 Myr. Moreover, the effect is larger for the longer periods so that it may be impossible to distinguish between the '41,000-yr period of the obliquity' and the '23,000-yr period of the precession' in the Precambrian times.

The accuracy of our results is limited by the accuracy of the values used: an increase of the lunar recession rate by 50%, which is still plausible for 440 Myr BP, reduces the astronomical periods by a further 10%. On the other hand, our conclusions hold for a more accurate astronomical solution¹³ as it seems that the astronomical frequencies considered here³, remain of the same order of magnitude in the new solution. The chaotic behaviour of the solar system may, however, challenge our results for the remote past¹⁴.

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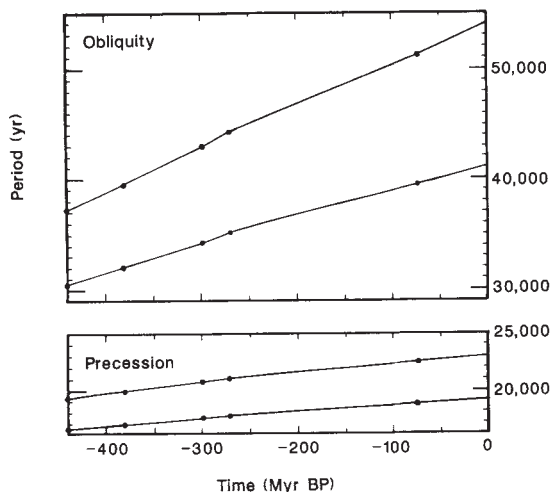
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Estimated values of the periods of the orbital parameters involved in the astronomical theory of palaeoclimates, considering the effect of the explicit variations of the Earth–Moon distance and of the Earth's figure and rotation. (Dots represent computed values from the data.)

DNA-binding domain ancestry

SIR—We would like to point out a similarity between the DNA-binding domain of the Myb family of proteins and homoeodomain-containing factors. This relationship indicates a common ancestry for the DNA-binding domains of these two classes of eukaryotic nuclear regulatory proteins, and predicts specific roles for parts of the Myb DNA-binding domain.

Genes similar to the *c-myc* proto-oncogene have been found in birds, mice, humans, *Drosophila melanogaster* and plants (*Zea mays*) (refs 1–6). The region common to the products of all these genes consists of imperfect repeats of 51–53 amino acids; in general there are three such repeats, except in *Z. mays*, where there are two, and in the two versions of v-Myb (encoded by the AMV and E26 viruses), which are truncated so that most of the first repeat is missing^{1,7}. These repeats are responsible for DNA binding⁸,

and it is assumed that the rest of the molecule is involved in transactivation of the target gene(s) of Myb (ref. 9).

In the Fig., *a* shows an alignment of the twenty known Myb repeat sequences, grouped as first, second and third repeats. (*Z. mays* sequences are best aligned if assigned as repeats 2 and 3.) Eighteen positions are highly conserved for all the sequences, an additional 11 positions being included if the occurrence stringency is lowered to greater than 60 per cent. Sixteen of these positions match with a homoeodomain (HD) consensus if two gaps are introduced (*b* in Fig.).

NMR spectroscopy of the homoeodomain from the *Antennapedia*-gene (*Antp*) product demonstrates three α -helical segments (*b* in Fig.) and confirms an earlier prediction that this domain is similar to bacterial repressors, with helices 2 and 3 forming the helix-turn-helix structure¹⁰.

The similarity between Myb and the homoeodomain is reinforced by a prediction of the secondary structure of the Myb repeat³ which suggests three α -helical

segments — designated here as Myb α 1–3. On the basis of conserved hydrophobicity and frequently occurring proline and glycine residues, the extent of the Myb α -helices can be estimated, revealing a close correspondence with the three helices of the homoeodomain¹⁰ (see *b* in Fig.).

As expected, the two alignment gaps fall within the loops interconnecting the helices. If these extrapolations are correct we would predict that the Myb α 2 and Myb α 3 helices would form a helix-turn-helix motif, with helix Myb α 3 making direct contact with bases in the DNA. Furthermore, the most conserved positions¹¹ in the bacterial repressor helix-turn-helix motif (which is related to the homoeodomain) align well with positions in the Myb consensus, especially those used in helix prediction (see *b* in Fig.). Applying a statistical algorithm derived from the bacterial sequences used to generate the helix-turn-helix consensus (ref. 11, with the modification suggested in ref. 12) to the homoeodomain consensus helix2-turn-helix3 region and the predicted Myb α 2-turn-Myb α 3 domain of the third repeat in chicken Myb (discounting the single insertion), gave scores of 1,363 and 1,358, respectively, compared with 1,675 for the λ Cro repressor and 1,336 for the λ CI repressor.

Myb sequences within a particular repeat grouping are most highly related at the C terminus, in the region of helix Myb α 3 (as expected if this is the intimate DNA contact point). A 'patch' of positive amino acids, as seen at the end of helix 3 in the homoeodomain, can also be seen at the end of the helix Myb α 3 in the repeat 3 sequences but not at the equivalent position in repeats 1 and 2.

A specific role for tryptophans in the Myb DNA-binding structure has been recently suggested¹³; it now seems likely that this may involve their hydrophobic character as, by analogy to the bacterial repressors, the tryptophans would be within a hydrophobic core.

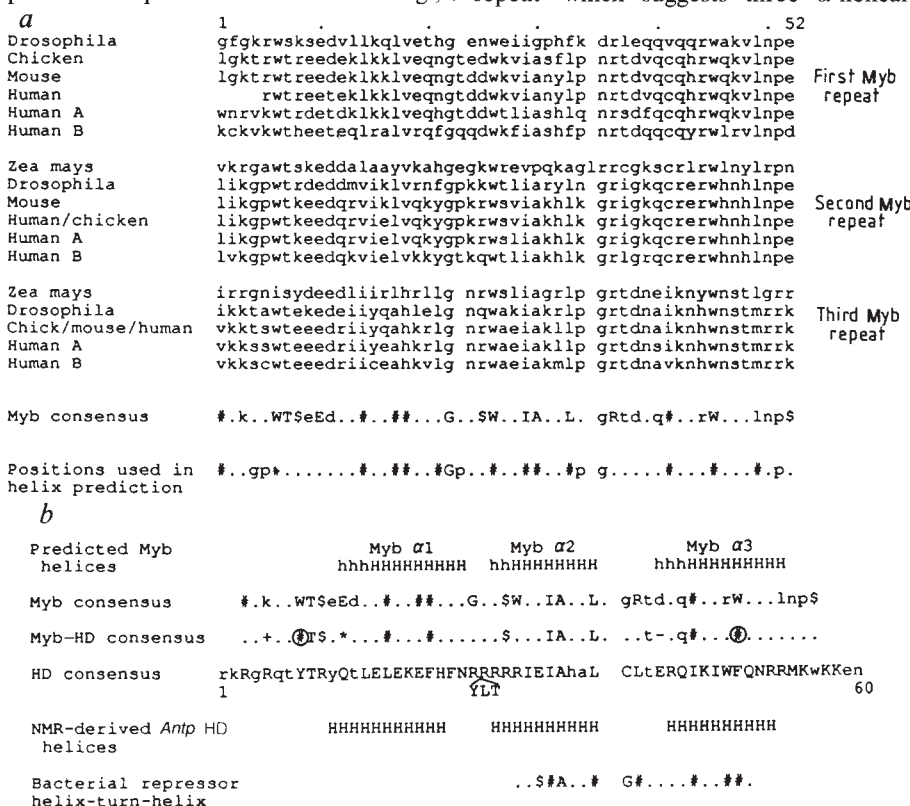
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a, Aligned repeats from Myb-related sequences^{1–6} (single letter amino-acid code), grouped as first, second and third repeats. The derived Myb repeat consensus is shown below (capitals and symbols, >85 % occurrence in the 20 Myb sequences, lower-case letters, residues which occur in >60 % of the sequences). Also indicated are the critical positions for helix prediction; conserved hydrophobicity (#), repeated every 3–4 residues, indicates an amphipathic helix; frequently occurring proline (P) and glycine (G) residues indicate interruptions. *b*, Derived information and comparison of the Myb consensus to the homoeodomain consensus and the bacterial repressor helix-turn-helix motif. The central line of the Fig. indicates the sixteen residues common to the Myb consensus and the homoeodomain (HD) consensus¹⁴. The extent of the experimentally determined and predicted helical segments are indicated by H (certainly helical) and h (possibly helical). The lower part of the Fig. shows a consensus for bacterial helix-turn-helix motifs derived from a compilation of 37 λ Cro-like proteins¹¹. Residues at conserved positions occur in >60 % of the sequences. #, hydrophobic; \$, large hydrophobic; \$, charged; +, positive; -, negative; *, E or Q.

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The political scientist

R. J. P. Williams

Linus Pauling: A Man and His Science. By Anthony Serafini. *Simon and Schuster*: 1989. Pp.310. £15.95. Published by Paragon House in the United States \$22.95.

LINUS Pauling is a tragic hero of the scientific age. His work on chemical bonding and secondary protein structure, amongst other contributions, means he ranks as a very great scientist. His coupling of the scientific analysis of nuclear reactions with pleas for political wisdom in the testing of nuclear bombs, and his fight for what he took to be a proper attitude to molecular medicine, were truly heroic. He had to put up with attacks from many scientific and non-scientific bodies, and was denied a passport because of his attitudes to bomb testing, branded a Communist and abused for his ways of looking at medicinal problems. He took great punishment and fought for his scientific views with a deep sense of social responsibility.

The flaw in all his activities lay in his unrelenting drive to succeed and his wish to be seen to be right while using what is euphemistically called the intuitive method of scientific investigation. From being a public figure of high stature with an idealistic philosophy, to being viewed as a lonely crank, is indeed a fall as great as in any classical tragedy. In this book Serafini has accumulated much of the evidence but in my opinion

he misses the central tragic point through a wish to defend Pauling, right or wrong, and a wish to promote the general idea of 'intuitive genius' in science. I wish first to pick up the threads of Pauling's life from the book and then to examine what his 'intuitive' thought really might be and the hidden dangers it holds.

The best place to begin is at the beginning of Pauling's life. Much though Serafini attempts to prove otherwise, he does not show that there was anything very remarkable in Pauling's early life as a schoolboy or as a student. He had a tough time, and he was bright, but there were many of his kind in the early part of this century. Serafini romanticizes when he sees a budding genius in the young Pauling, because he always uses as evidence

happenings in a later period of his subject's life.

It was only after his visit to Europe (to Sommerfeld) in 1926 that Pauling came into the limelight. He saw the application of valence bond and ionic theory to chemistry. At the same time as generaliz-

IMAGE
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FOR COPYRIGHT
REASONS

Happy days — Linus Pauling being congratulated by his family after receiving the Nobel prize for chemistry in Stockholm, 1954.

ing his views in brilliant fashion he made many telling smaller observations (his summarizing book, *The Nature of The Chemical Bond*, is a masterly work). But already he was digging the trap into which he would fall. He overlooked or chose to ignore the work of Bragg on ionic crystals, he undoubtedly ignored molecular orbital and crystal field theories, and he brushed aside the physicists' quantitative approach to metals. Not only was he building his own reputation but he was making enemies and taking risks. Switching to protein structures, he uncovered the α -helix, a most valuable piece of work, but at the same time ran into pitched battles with those who did not accept his views. It happened that he was 90 per cent right,

and when I first listened to him in Oxford in 1948 he was without doubt a great scientist indeed. But how had he achieved this greatness? Through intuitive genius? In my opinion it was through imagination, a deep factual knowledge and very hard work indeed.

In the late 1940s, Pauling became interested in nuclear fall-out from atomic weapons. Here the facts were fewer, the scope for different viewpoints greater and the political consequences of his work immeasurably more significant. At about the same time the logic of socialism attracted both himself and his wife more strongly. Pauling used his skills as a scientist and as

an advocate to attack the government of his own country and the scientists who opposed him. As before, he also used his imagination. Believing more and more intensely in himself, he fought for what he saw to be a rational way to peace. It was an admirable fight, but looking at it with hindsight, as one can with Serafini's book in hand, one can see the hazards. What was the evidence really like? Do the facts alone support the conclusions, or must one believe first in a politically wise structure of one's own making? The trap for the egocentric idealist is ever present.

In part, the over-reaction of the authorities in the United States probably saved Pauling at this stage, and in large measure his stature increased. Then the political battle for peace was won by Kennedy, while we all held our breath, using the opposite route of speaking from strength. The antagonism that was developing all the

time towards Pauling can be seen by the way in which the award of the Nobel Peace Prize to him was greeted in a most unpleasant manner in the United States. At the end of this period, Pauling had fewer friends and many powerful enemies who cast doubt on both his politics and his scientific insight.

Pauling next turned to a very different science, medicine, preaching that a molecular approach was what was needed. In this general respect he was right, but unfortunately he chose to espouse a particular cause — that of vitamin C as the preventive medicine against the common cold and (a view he adopted later) against cancer. Here there were few facts and many hypotheses. Pauling's statements

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were full of sound and fury, but his ideas were of unknown and untested (some would say untestable) significance. Colleagues distanced themselves from him. His wife, who was taking vitamin C, died of cancer. The first paper of an academician to be refused by the *Proceedings of the National Academy of Sciences* was his. He dismissed his colleagues. Instead of seeing tragedy in all of this turmoil, Serafini tries to hold out possibilities that Pauling was always right in the way he attacked problems. How could things be otherwise if he was an intuitive genius?

In my opinion, the greatness of Pauling as a scientist lay in his ability to use facts imaginatively and constructively, with considerable theoretical insight but in at best a semi-quantitative manner. It is the way in which many great chemists and biologists work, and it succeeds as long as there is a sufficiency of fact to guide and limit the imagination. Where the method fails is when the facts are few and one's choice of hypothesis cannot be much more than speculation. The only proven scientific method then is to construct a quantitative theory, to get more facts and to prove the theory valid. When Pauling drifted away from his detailed physico-chemical background towards biology he had already built his belief in his intuition, but afterwards he had too few facts to support his thinking. He made errors in the structure of DNA and the atomic nucleus, and, I believe, in his views on preventive treatment for certain diseases. Often his approach can only be described as a consequence of a reckless wish to be in the picture. The flaw of self-belief, perhaps even a cult of personality, enveloped him.

Scientists appear to need the image of 'intuitive genius', as espoused here by Serafini, and many propel themselves towards this reputation. Rather than believe in steady progress, which is the principal characteristic of science if its quantitative methods are applied thoroughly and with skill, they think of their individual 'breakthrough' as having been generated by some personal artistic insight. This is an illusion encouraged by the modern theatre of prizes and conferences, created by scientists and loved by the media. Only too often the very best of those who are imaginative and hard working rise to the top only to become in later life dreamers attempting conquests in areas outside their skills. Much though Serafini tries to promote the image of the scientific genius, there may be no such thing and this very image may well have been the trap into which Pauling fell. Pauling, in my opinion, was for quite some time just the best of us. I know of no higher praise. □

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Flaws of nature

John D. Barrow

Reading the Mind of God: In Search of the Principle of Universality. By James Trefil. Scribner's, New York: 1989. Pp. 232. \$18.95.

MIND-reading is a racket. By foreknowledge and subtle suggestion one can peddle one's own thoughts as those of the subject. Reading the mind of God is therefore likely to be an exceptionally tricky business.

Perhaps the fact that the enterprise is doomed to failure from the outset is reason enough why this book fails to live up to the author's intentions and those of the publisher. It claims to be a popular study in the philosophy of science focused upon the 'principle of universality' (that is, the principle that the laws of nature which we discover here and now hold sway in the Universe everywhere and everywhen). But at one level I found the book to be a mystery, the mystery being the whereabouts of any real discussion of its stated subject. Occasionally, after a description of a piece of physics — the behaviour of light, the saltiness of the sea or the age of the Earth — the author remembers what he had hoped to write about and adds a few sentences of homespun philosophy about the laws of nature. But that's about it.

Because of this idiosyncrasy, I suggest that the prospective reader should wipe clean any thought of the subtitle and simply enjoy the separate chapters. They are accurate and interesting in their explanation of scientific ideas, some of which rarely find their way into popular science books.

Particularly engaging is the story of Fraunhofer's early life as an industrial glass caster, which culminated in his becoming the most skilled nineteenth-century manufacturer of precision scientific instruments. This account leads naturally into the birth of spectroscopy, new astronomy and the discovery of helium in the Sun (in which, incidentally, Sir Joseph Norman Lockyer, the founding editor of this venerable periodical, had a part). Similarly there is a successful cameo about the turn-of-the-century dilemmas regarding the age of the Earth and battle between the physicists, biologists and geologists over the relevant evidence. We learn about Kelvin's genius for practical things, and his design of the modern fireplace, but all too little about the influence of his religious views and the role played by attitudes towards darwinian evolution.

Here, and elsewhere in the book, Trefil has one eye upon modern creationist arguments about the antiquity of the Earth and the uniformity of nature. What could have been made clear is that the point of dis-

agreement is a subtle one for the layperson. The creationists interpret the uniformity of nature to mean a constancy in the events of nature whereas Trefil, like most other scientists, lays stress upon the constancy of the underlying laws of nature. This constancy does not require any uniformity in the outcomes of those laws. Indeed, in some of his passing comments on universality Trefil fails to appreciate the importance of this point. It is not enough, as he argues, to know the laws of nature in order to understand the structure of the Universe and reconstruct its past from the present. Even if we knew the initial conditions, we would still be faced with restoring the past symmetry-breakings which determine the particular outcomes of the laws of nature that we witness. Knowledge of the laws is necessary but not sufficient to understand the structure of the Universe.

Another point that would trouble the alert reader is what to understand by the much-vaunted invariance of the laws of nature. If Trefil has in mind the 'real' laws (assuming that such things exist) then by definition they cannot be changing — one can always re-express a proposed law of change as the constancy of some higher derivative of it. If, on the other hand, he simply means our current theories, then these 'laws' can of course be proven wrong. Quantities that are supposed to be constant may be found to change. These are not changing laws, of course, simply wrong laws. In a book for this sort of audience it is necessary to make clear which picture one is adopting — are the laws of nature descriptive accounts of what has always happened (and so cannot by definition be broken, as the creationists' defence of the miraculous sometimes argues), or are they prescriptive (and so can be found to be 'broken', but only in the undramatic sense that they were not the correct laws).

The closest Trefil gets to tackling questions of this sort is in his helpful demarcation of the scientific method into the two processes of theory creation and theory verification or falsification. Sociologists of science lay great stress upon the former element, which is partially subjective and influenced by all manner of extracurricular prejudices, whilst the exasperated scientist invariably points the sociologist towards the objective and hard-nosed activity of theory testing.

Reading the Mind of God exhibits good small-scale structure. The individual sections and even chapters are stimulating and will be instructive to students and non-specialists alike. But the book possesses no coherent large-scale structure that relates in any helpful manner to its title and the author's overall intentions. God can rest easy. □

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The problems of a dinosaur

Dale A. Russell

Dynamics of Dinosaurs and Other Extinct Giants. By R. McNeill Alexander. *Columbia University Press*: 1989. Pp. 167. \$30.

It is not easy to write simply, clearly and concisely. R. McNeill Alexander is a master of the art, as he shows in this charmingly quantitative volume which deals primarily with the biology of dinosaurs.

Many of those interested in dinosaurs (including myself) lack an adequate background in the principles of physics and engineering as they apply to vertebrate morphology. Those principles are demonstrated in a sampling of topics including assessments of the weights of dinosaurs, their motion as revealed by trackways, their 'athletic' ability, the structure and function of the backbone, reproductive competition and metabolism. Flight in pterosaurs and swimming in giant marine reptiles are also considered, as is the controversy surrounding the extinction of the dinosaurs and subsequent gigantism in mammals and birds. The text is all the more valuable for its open-minded and non-polemical tone.

Many stimulating insights emerge from the book. Among those I particularly appreciated are the comments on the ability of sand and wet clay soils to support weight; the apparent lack of athleticism in brontosaurs and in *Tyrannosaurus*; the use of the tail in kangaroos to offset angular momentum generated by the legs in hopping; the small size of the horns in *Triceratops* relative to horn-body-weight trends in antelopes (were *Triceratops* jousts correspondingly less energetic?); the prediction of a neck 'crumple zone' to absorb the shock of head-to-head impact between two colliding dome-headed dinosaurs; the suggestion that duck-billed dinosaur females (like human females?) tended to prefer mates with deeper voices; and the physical reasons for the importance of aspect ratio to fluid dynamics.

There are, however, points to disagree with. For example, the vertebrae in the brontosaur neck are extraordinarily pneumatic, and the specific gravity of the neck may well have been less than that of the body, contrary to what Alexander implies. Indeed, the neck was often partly or completely separated from the body upon burial, possibly because of its relative buoyancy (and, perhaps, because of the absence of a single, powerful neck tendon). In the book, no conclusions are drawn with respect to 'hot-bloodedness' in dinosaurs; among the various kinds of

evidence cited, however, predator-prey ratios as preserved in the fossil record are said to be probably the most significant. In the Cretaceous assemblage from Canada (Oldman Formation) that Pierre Béland and I have examined, the ratio happens to suggest that dinosaurian carnivores had metabolic levels less than those of mammals. I would agree, though, with the suggestion that, in the case of the 'cold-blooded' alternative, the standing crop of large dinosaurian herbivores

might well have been spectacular.

Many fascinating problems suggest themselves from a thoughtful perusal of this book. Specialists should read it, reflect on it and let Professor Alexander have their ideas for further research. Those disparate disciplines, physics and palaeontology, have much to offer each other. □

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Stan Ulam

David K. Arrowsmith

From Cardinals to Chaos: Reflections on the Life and Legacy of Stanislaw Ulam. Edited by Necia Grant Cooper. *Cambridge University Press*: 1989. Pp. 320. Hbk £40, \$75; pbk £15, \$24.95.

I SUSPECT that, like me, most mathematicians have heard of Stanislaw Ulam but have come across his name in connection with no more than one or two mathematical disciplines. With this prejudice, I felt that Ulam's contributions to mathematics were not great enough to warrant such a weighty, large-format book as this. I was wrong — the number of areas in which Ulam produced original work is quite surprising, and it would take a person with very wide interests fully to appreciate the range of his endeavours.

There are three parts to the book. In the introductory section, Ulam is known to everyone as Stan and the reader gets on first-name terms with him too — appropriately so, for it is always good to know what drives the leaders and visionaries in any academic subject and how they go about their lives away from the formality of the written paper. Fellow mathematician Gian-Carlo Rota, Ulam's close friend, is responsible for much intimate insight and sets the informal tone with an anecdotal article which gives an appreciation of Ulam's personal and scientific qualities. After several other revealing and fond tributes, part two gives an appreciation of his scientific legacy. The layout is attractive and the articles are littered with sketches and pictures as well as reproductions of letters from von Neumann to Ulam, his colleague. The unpredictable, even chaotic, bitty format makes for enjoyable reading.

The scientific articles are well written in accessible style. They testify to Ulam's crucial and often early intervention in many key research areas, after which he generally left others to flesh out the ideas. Several papers contain beautiful colour plates. Together with the book's great bulk, these suggest that *From Cardinals to Chaos* should be put out for browsing

alongside other large-format, glossy volumes. Yet a glance at the index shows that it contains plenty of mathematical detail and the contents are much more demanding than the initial appearance suggests.

The overwhelming scientific impression is of a strong cross-fertilization between the various strands of Ulam's interests in science, particularly physics, and mathematics. Some of the titles of the papers — "Strange Attractors and Number Theory", "Ergodicity and Biomathematics", "Non-linear Sciences", "Molecular Genetics" and "Turbulence" — testify to his wide-ranging vistas.

The last part, entitled "The Ulam Touch", is short but highly entertaining, and consists of hitherto unpublished items. It says much about Ulam the man. We should be grateful that Rota has transcribed some of his conversations with Ulam on general philosophical points as well as discussions about some of his most famous acquaintances such as von Neumann, Erdős, Teller and Gamow. There are also touches of hilarity, with a "memorable memo", and a "Dialogue" written by Ulam which parodies the arguments of both the pro- and anti-nuclear lobbies around him during his time at Los Alamos. The "Dialogue" was described by Ulam as a "top-secret skit", not meant for public consumption, and that "posterity should decide".

Anyone who reads this attractive and highly informative book is likely to conclude that Ulam was a scientist of great originality, and that posterity will acknowledge his many seminal contributions. □

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New in paperback

- *The Age of Gaia* by James Lovelock. Published by Oxford University Press, £4.95. See review in *Nature* 336, 270 (1988).
- *The Mathematical Tourist* by Ivars Peterson. Published by W. H. Freeman, £9.95, \$10.95. Reviewed in *Nature* 336, 292 (1988).
- *The Purpose of Forests* by Jack Westoby. Published by Basil Blackwell, £12.95, \$25.95. Reviewed in *Nature* 330, 286 (1987).
- *Racial Hygiene: Medicine Under the Nazis* by Robert N. Proctor. Published by Harvard University Press, \$14.95, £11.95.

Making good

Jack Young

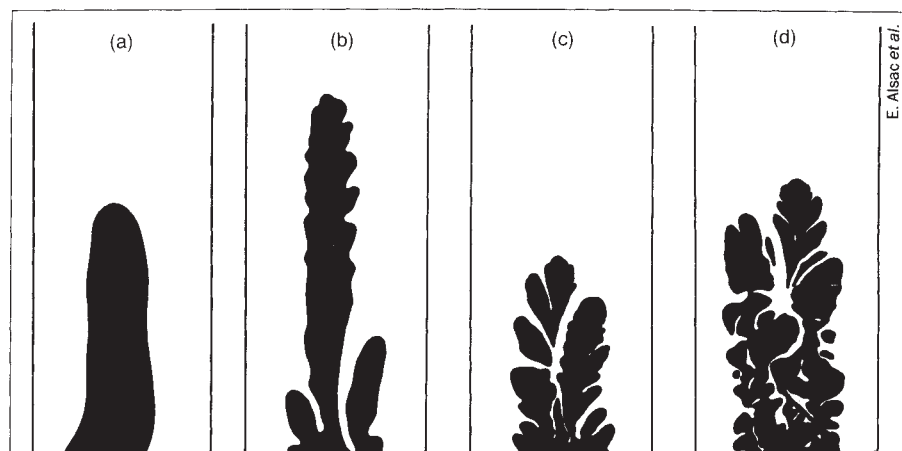
The Causes and Cures of Criminality. By Hans J. Eysenck and Gisli H. Gudjonsson. Plenum: 1989. Pp.309. \$41.40.

CRIMINOLOGY is an interdisciplinary field *par excellence*, demanding the integrated skills of the psychologist, sociologist, lawyer, human geographer and statistician. As Eysenck and Gudjonsson say, a "new wind is blowing through these fields, driving away the miasmas of ideological preconceptions and the critical biases" and "criminology is becoming a science, rather than being a football kicked about by ideological partisans of one persuasion or another". But they show little awareness of this new wave of thinking. Rather, they present us with the 'old wind' of psychological reductionism masquerading as a gust of innovation.

Crime involves an objective action and a subjective evaluation of that action as criminal. Over time, between different groups of people and in different countries, the evaluative element varies. What was considered the normal, perhaps necessary, chastisement of children in Victorian times, would be considered child abuse today. What is permissible physical punishment of children in Britain would be illegal in Scandinavia. This essential dyad — behaviour and the differential reaction to it — is simply ignored by the authors; it is as if criminal statistics had an independent existence outside of the members of the public, judiciary and police force, who, of course, necessarily exercise discretion in their adjudication. As a result, they present us with a graph of the rise in violent crime in Britain between 1970 and 1980 as if it were an objective piece of data.

This simple graph demands a complex of explanation involving an analysis of the causes of the changes in violent behaviour, in police-public relations (that is, willingness of people to report incidents) and in levels of tolerance of crime. Yet the authors present it as an indictment of 'current' sociological theories of crime, which they characterize as presuming an inverse linear relationship between absolute levels of wealth and the crime rate. But that is just what has not happened — crime rates have risen with the increase in wealth. True, such beliefs were current amongst positivist criminologists in the 1950s but it is remarkable that Eysenck and Gudjonsson should choose to take issue with already debunked theories.

The authors' distaste for subjective factors is further exemplified in their discussion of the relationship between relative deprivation — a key concept in criminology — and crime. Social dis-



Sticky fingers — instabilities in 'viscous fingering' processes can lead to a variety of patterns developing in the advancing medium. In the figure, the dark region is an air bubble which is being forced by low pressure into a dilute, aqueous colloidal suspension of clay. As the driving pressure increases, from left to right, interfacial instabilities cause disruption and break-up of the air 'finger'. The figure is taken from *The Fractal Approach to Heterogeneous Chemistry*, edited by David Avnir, published by John Wiley and Sons.

content, and in specified conditions crime, arises where there are perceived injustices in the distribution of wealth and status. The subjective perception of objective disparities often becomes greater when actual differences in wealth narrow. A caste society with wide differences of social position sanctified by tradition can exhibit little relative deprivation. In contrast, a society such as our own, which claims to be meritocratic and which has considerably less differentials of wealth, yet palpable injustices in reward, can generate extremely high levels of relative deprivation. Eysenck and Gudjonsson, with their obsession with the 'objective', present us with a graph indicating a narrowing of disparities accompanied by a rise of crime as if it were an indictment of relative deprivation theory.

Again, in their account of the relationship between individual constitutional and psychological differences, and the propensity to criminality, the authors persist in engaging in a reductionism which severely underestimates social and cultural factors. For example, marginalized groups of working-class youths react to exclusion from economic and social status by generating subcultures which emphasize hedonism, excitement and status achieved through aggression. It is, therefore, quite unremarkable that offenders score highly on psychological tests such as Eysenck's introversion-extraversion scale. For all of the questions enshrined in testing for high extraversion reflect precisely these values.

As far as constitutional factors are concerned, more muscular people (mesomorphs) are, of course, more likely to commit violence than fatter endomorphs; violence is a currency predicated on stronger individuals acting aggressively towards those who are weaker. The question is why certain mesomorphs in particular parts of the social structure, and in certain countries rather than others,

engage in violent behaviour. The repeated presentation of a correlation between the mesomorphy of offenders and crime just discovers the obvious and elevates it to the level of the causal.

As a sociologist, arguing against behaviourist dogma and psychological reductionism, I would not wish to deny the effects of individual differences, both of psychology and constitution. Too often our discipline has denied the individual. Yet Eysenck and Gudjonsson commit what Elliott Currie calls the 'fallacy of autonomy' — they reduce behaviour to individual free-floating traits and are unable to understand how the asocial is a product of the social.

The authors move from analysis of the cause of criminality to its cure. They point, quite correctly, to the absurd fashion in which governments throw money at the problem but rarely monitor the results. Yet when we come to Eysenck and Gudjonsson's prescriptions for reducing crime we find a catalogue of the commonplace — longer prison sentences for persistent offenders, increasing the likelihood of detection of crime, increasing prison accommodation, and greater discipline in the family and the school. The prescriptions are, as they put it, "very much in line with common sense". Yet such common sense notions have been tried *ad nauseam* and have palpably failed. A scientific criminology must be based on a realism that cuts through the gut reaction of punishment; that understands that the 'punitive obsession' itself is a product of an unequal society; and that seeks to generate solutions based on the social and individual causes of crime which are well researched and adequately monitored. □

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Annihilation of ecosystems by large asteroid impacts on the early Earth

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Large asteroid impacts produced globally lethal conditions by evaporating large volumes of ocean water on the early Earth. The Earth may have been continuously habitable by ecosystems that did not depend on photosynthesis as early as 4.44 Gyr BP (before present). Only a brief interval after 3.8 Gyr exists between the time when obligate photosynthetic organisms could continuously evolve and the time when the palaeontological record indicates highly evolved photosynthetic ecosystems.

THE early Earth was bombarded by large projectiles at the same time that large impact basins formed on the Moon. It is sometimes asserted (for example, ref. 1) that these impacts would have precluded the continued existence of life before 3.8 Gyr, but little has been done until recently to quantify the untoward effects of impacts on life. Maher and Stevenson² considered the possibility that frequent impacts could have prevented the origin of life, particularly for sites at the surface. They did so by comparing the time between impacts at a given location on the Earth's surface with the time required for biogenesis. In this paper, we concern ourselves with a related, yet inherently different problem, namely: When did the last planet-sterilizing impact occur? That is, we assume that life originated in some unspecified manner, and then consider what it would have taken to have wiped it out. This approach is better because the time-scale for originating life is very poorly constrained, whereas early ecosystems with well defined organisms are broadly constrained by analogy with modern ones. In addition, extant organisms and the palaeontological record relate only to continuous evolution after the last impact to sterilize the planet.

We argue that the major way in which the planet can be sterilized is by evaporation of ocean water. This process affects some organisms more strongly than others, depending on how much water is evaporated and what type of ecosystem is envisioned. Certain classes of ecosystems may be able to withstand thermal perturbations lasting hundreds or thousands of years. We first discuss the flux of impacting objects, then the effects of impacts, and finally their effects on plausible early ecosystems.

Flux of impacting objects on the early Earth

The impact of a Mars-sized object, which is believed to have formed the Moon, would have either melted or vaporized much of the Earth³. Neither living cells nor complex organic molecules would have survived this blow. It is the flux of lesser later projectiles on the early Earth that is relevant to biology. No direct record of the earliest impacts exists on the Earth. By contrast, the lunar record is well preserved and well studied. Thus, the lunar crust is a reasonable starting point for our study. Our approach follows that in refs 4–7. We represent impact fluxes in terms of the equivalent thickness that the projectiles would add to a planet if all the material were retained. This measure is independent of planetary size so long as gravitational

focusing and the statistics of the small number of largest objects can be neglected.

Geochemical estimates of flux. A minimum estimate of the material that hit the moon is obtained from the meteorite component retained in lunar samples. Rocks exposed in the lunar highlands, which are believed to be typical of the lunar upper crust, are breccias composed of pristine rocks, impact melts and still older breccias. It is generally agreed that Ir abundances indicate a meteoritic component of 1–2%. It is controversial whether Ni abundances indicate a comparable meteoritic component that lacks Ir (ref. 8) or an internal component that was reduced to metal in the crust⁹. Studies of lunar meteorites indicate that the excess nickel is local to the nearside landing sites and that the meteoritic component is between 1–2%¹⁰. The total thickness of lunar crust that is contaminated with siderophiles is not well constrained. Granulites believed to be deeply buried breccias as old as 4.26 Gyr (ref. 11) and 4-Gyr old near-surface breccias are both enriched in siderophiles, indicating that the meteoritic component is mixed in much of the upper crust. The megaregolith thickness is one estimate of the mixing depth. Spudis and Davis¹² give half the thickness of the lunar crust, 35 km. If the meteoritic component is between 1% and 4%, this implies a total meteoritic thickness between 0.35 km and 1.4 km, with 0.7 km being our preferred estimate.

The time of these impacts is also constrained. If a magma ocean existed on the Moon, the upper crust should have frozen from the top down while being stirred by impacts⁷. The date at which a mostly solid crust existed at any depth defines the start of the time interval for Ir and Ni retention in the lunar regolith. The oldest lunar rocks approach 4.5 Gyr and indicate that a crust existed early in the history of the planet¹³. The upper part of the lunar crust, ferroan anorthosite, solidified as early as 4.44 Gyr (ref. 14). The lunar crust was largely solid by 4.36 Gyr (the model age of KREEP (potassium rare-earth elements phosphorus) basalt), which is believed to be the date of the last solidification of the base of the crust¹⁵. Much of the accretion was over by 4.26 Gyr, the age of the oldest regolith sample.

Energy and mass of estimates of total projectiles. The lunar crust is so mildly churned that this fact by itself sets a useful upper limit on the flux of large projectiles. Large impacts were sufficiently rare that regional heterogeneities, primary stratification, and even local igneous bodies have not been obliterated^{16,17}. Impacts similar to or larger than Imbrium seem to have been rare after the upper crust froze at 4.44 Gyr, as the crustal stratification remained intact over much of the lunar surface. In particular, impacts larger than Imbrium should have excavated mantle, but no lunar mantle samples have ever been found despite careful search. Similarly troctolite, an expected constituent of the lower crust which was excavated by Imbrium, is quite rare¹⁸. The total amount of material from impacts occurring since 4.44 Gyr should be less than the 1.88-km equivalent layer needed to saturate the surface with Imbrium-sized craters⁴.

The size of the largest projectiles is relevant in this regard (Fig. 1). The energy of Orientale (diameter, 930 km), the youngest (3.8 Gyr) and best preserved of the basins, is obtained from the amount of buried heat by examining the thermal contraction of the centre of the basin¹⁹. If we assume that 25%

of the energy is deeply buried, the impact energy is between 4×10^{25} J and 3×10^{26} J with the middle of the range 1.2×10^{26} J being our preferred estimate. This corresponds to an object of mass 1.4×10^{18} with a diameter of 93 km (assuming a projectile density of $3,300 \text{ kg m}^{-3}$ and a velocity of 13 km s^{-1}). The ejecta mass²⁰ of 2.4×10^{19} kg implies a ratio of ejected mass to projectile mass of 17. The largest confirmed basin, Imbrium, ejected 3.6×10^{19} kg (ref. 21) or 1% of the 35-km-thick megaregolith layer. The impactor mass was 2×10^{18} kg (ref. 6) Baldwin⁴ gives an equivalent thickness of 0.2 km of accretion after 4.30 Gyr, equal to the mass of 11 Imbrium projectiles.

Extrapolating to the Earth. The Earth should have been hit with many more projectiles than the moon as it has more surface area and larger gravity. Statistically, the Earth is also likely to get hit with larger objects. For the impact velocities we assume below, the Moon is hit by $\frac{1}{24}$ of the objects and the Earth the remaining $\frac{23}{24}$. The probability that the moon is not hit by any of the 16 largest objects is over 0.5, because $(\frac{23}{24})^{16} > 0.5$. Thus, if Imbrium is the largest lunar projectile after 4.30 Gyr, 16 larger objects are expected to have hit the Earth in the same period. The time and size of these impacts are difficult to constrain because of the small numbers involved. For example, the late impact of the Imbrium object on the Moon could be a statistical fluke. There is no convincing evidence for a 'spike' of late impacts between 3.8 Gyr and 3.9 Gyr and no obvious physical mechanism for producing them⁶. Conversely, it can be concluded that the number of huge objects that hit the Earth was not large.

Theoretical size-number distributions are one way to infer the size of the large objects that hit the Earth from the lunar record. For fragmentation, such mass distributions are power laws. For example, the cumulative number of objects with a mass greater than m is proportional to m^{1-q} . We can visualize the properties of such a distribution by 'binning' the objects in logarithmic intervals of mass. For $q = 2$, there is equal mass in every bin. For $q = \frac{5}{3}$, there is equal surface area in each bin, and most of the mass is concentrated in the largest objects. The largest is expected to be $2 - q$ of the total mass of the ensemble. With some algebra, it can be shown that the median fraction of the total mass hitting the moon is $(\frac{1}{23})^{1/1-q}$. The size distribution is maintained in a quasi-steady state by a fragmentation cascade. Theoretically, expected values of q range from $\frac{5}{3}$ for mild collisions to 2 for violent collisions²².

The most relevant surviving class of objects for impacting the early Earth are asteroids with diameters between 130 and 260 km which are believed to be fragments of larger bodies. Such bodies would enter Earth-crossing orbits after the fragmentation event. The lower end of the range is comparable to the Imbrium object from which our extrapolation starts. For this size range, $q \approx 2$ (refs 22, 23). If this distribution applies, the largest objects hitting the Earth need not have exceeded 260 km.

The larger gravity of the Earth increases the probability and energy of impacts²⁴, by a factor of 1.74 assuming an approach velocity of 13 km s^{-1} , which corresponds to an impact velocity of 13 km s^{-1} on the Moon and 17 km s^{-1} on the Earth. The approach velocity (weighted for probability of impact) of present Earth-crossing asteroids, 16.8 km s^{-1} (calculated from ref. 25, Table 8.5.1), is higher for large early asteroids by an unknown amount because the sample includes small asteroids that began as comets²⁶ and small asteroid fragments ejected by collisions²⁷.

Global sterilization by large impacts

We are concerned here with global effects of large impacts which would sterilize the entire planet. Boiling of the ocean by the heat of rock vapour produced by the impact is the most obvious such effect. Other global effects, including pressure changes in the ocean from large tsunami waves, fouling the ocean with meteorite debris and ejecta, and salinity changes as the ocean is boiled off and later as the water rains out have no obvious planet-sterilizing effects and are not considered further. The

crater itself is of only regional importance. For example, according to the scaling relations compiled by Maher and Stevenson², an asteroid of 500-km diameter moving at 17 km s^{-1} would create a crater 1,500 km in diameter with 0.1-km-thick ejecta extending over a region 4,000 km in diameter. The ejecta at the rim of this crater would be 3 km thick.

Energy considerations. The estimates of the energy necessary to boil the ocean or the photic zone are insensitive to whether the final state is considered to be isothermal, adiabatic or on the vapour-saturation curve. For water that is initially at 0°C , isobaric boiling requires $2.5 \times 10^6 \text{ J kg}^{-1}$ at 1 bar and $2.1 \times 10^6 \text{ J kg}^{-1}$ at the critical pressure. Thus, $\sim 4 \times 10^{26}$ J of energy are needed to boil the 200-m-thick photic zone of the ocean, and 5×10^{27} J is needed to evaporate the entire ocean (1.4×10^{21} kg) and raise the temperature of the water vapour above the critical point. To further raise the surface temperature to the melting point of typical silicate rocks requires about half again as much energy.

An impact of an ocean-evaporating scale corresponds to a 440-km diameter (an object of mass 1.3×10^{20} kg)—roughly the size of the large asteroids Vesta and Pallas—hitting at 17 km s^{-1} . A 190-km-diameter (mass 1.1×10^{19} kg) object would evaporate the photic zone. We estimate that $\sim 25\%$ of the impactor energy goes into evaporating sea water, 25% is radiated to space, and 50% buried near the impact site. For impacts of this size, most of the material that leaves the crater is either melted or vaporized²⁴. Rock vapour is produced directly by the impact and secondarily when fine particles of ejecta return to the atmosphere. Thus, the planet is quickly enveloped by about 100 bar of hot rock vapour and suspended droplets. The rock-vapour atmosphere radiates upwards to space and downwards onto the ocean with an effective temperature of 2,000 K. For an impact on an ocean-vaporizing scale, the radiative cooling time for rock vapour to condense is a few months. The precise radiating temperature is not too important, because roughly half of the rock vapour's energy is radiated to space, and roughly half is absorbed by the ocean.

To continue with the events following impact, the high opacity of sea water to infrared radiation concentrates boiling to a thin surface layer. Radiation from the rock vapour ablates the surface of the ocean, leaving the ocean depths cool. Convective mixing of the ocean is strongly inhibited by the temperature gradient. As water vapour is somewhat transparent to 2,000-K blackbody radiation, the oceans are not effectively shielded from the hot rock above, yet the water vapour is sufficiently opaque that it is quickly heated to the 2,000-K atmospheric temperature²⁸. During this period the atmosphere is kept roughly isothermal around 2,000 K, the condensation temperature of silicates. Water clouds would not form under these conditions.

Once the rock vapour has condensed, a steady-state water-vapour atmosphere above a liquid ocean can radiate to space no faster than a certain threshold level that is only some 30% higher than Earth's present infrared flux²⁸. This is precisely the 'runaway greenhouse' threshold often encountered in the comparative planetology of Earth and Venus²⁹. This rate is determined by the transmission of infrared radiation through a moist, convective, H_2O -dominated atmosphere. The presence of clouds can only reduce the rate of thermal emission. Faster radiative cooling demands very high surface temperatures, $\sim 1,500 \text{ K}$, to exploit the lower opacity of water vapour to visible and near-infrared radiation. The runaway greenhouse threshold also controls the maximum rate at which a water-vapour atmosphere can cool while water condenses.

For the minimal ocean-vaporizing impact, about half the ocean remains as liquid water at the time when the rock vapour has rained out. (About 300 m of rock raindrops would sit on the ocean floor.) At this point the atmosphere would consist of about half an ocean (140 bars) of hot ($> 1,500 \text{ K}$) steam. Shortly afterward, the top of the steam atmosphere cools sufficiently to form a moist, convective, optically thick upper layer. Thereafter,

the planet radiates to space or below at the runaway greenhouse threshold, which is negligible compared with the rate at which energy is transferred to the ocean. In the minimal ocean-vaporizing impact, the last droplet of ocean is evaporated as the first droplet of rain reaches the ground. The surface temperature is then near the critical temperature, 647 K.

The lifetime of the steam atmosphere can be estimated by dividing the energy invested in steam by the effective cooling rate of 150 W m^{-2} , this being the difference between the runaway greenhouse threshold and total absorbed solar irradiation around 4 Gyr BP. The cooling time is on the order of 2,000 years for a minimal ocean-vaporizing impact. (The cooling rate is twice as fast if the clouds are sufficiently opaque that no solar radiation is absorbed; this difference is unimportant here.) An impact $\sim 50\%$ more energetic than the minimum ocean-vaporizing impact will leave a 1,500-K steam atmosphere when the ocean evaporates. This takes 3,000 years to cool. Cooling times for still larger impacts may not be greatly longer, because the runaway greenhouse threshold does not apply for very hot surfaces. But it is clear that extremely adverse conditions are expected for at least 3,000 years after any impact even modestly larger than the minimal ocean-vaporizing impact.

By contrast, adverse conditions in the atmosphere and the shallow ocean persist for about 300 years for an impact which evaporates only the photic zone. Bottom-dwelling photosynthetic organisms would be killed directly as the ocean boiled away above them. Floating photosynthetic organisms might, if they were lucky, be continually mixed downward as the ocean surface boiled. Survivors would, however, be killed when they were mixed back up to the surface and exposed to hot, fresh rainwater.

Discussion and uncertainties. Given that 25% of the impact energy is used to evaporate sea water, a total impact energy of $2 \times 10^{28} \text{ J}$ is needed to evaporate the entire ocean. An object of mass $1.3 \times 10^{20} \text{ kg}$ (440-km) hitting the Earth at 17 km s^{-1} would suffice. Ocean vaporization is plausible at much later times given a scale-invariant impactor distribution. Using the inference that

the Imbrium projectile is $\frac{1}{11}$ of the mass colliding with the Moon after 4.3 Gyr to obtain $q = 1.91$, the largest object inferred to hit the Earth since 4.44 Gyr is expected to account for 9% of the total accreted mass, or $3 \times 10^{20} \text{ kg}$. Between 3.8 and 4.3 Gyr, an object 31 times as massive as the Imbrium projectile, or $6 \times 10^{19} \text{ kg}$, is also statistically expected (Fig. 1). Given the uncertainties inherent in such a scale-invariant distribution, the last ocean-vaporizing impact may have occurred as early as 4.44 Gyr or as late as 3.8 Gyr. Impact vaporization of the photic zone as late as 3.8 Gyr is probable, however, whatever the size distribution, as the larger fragmental asteroids would suffice. A long time interval between the last impact to vaporize the ocean and the last to have evaporated the photic zone is thus statistically likely.

Survival of ecosystems

Biological systems require an energy source. The present biosphere receives its enthalpic buildup largely from solar energy; a second minor component in the deep sea is derived from the oxidation of reduced components at hydrothermal vents. The latter depends on oxidants being produced in the photic zone. It is likely that both these energy sources were available on the early Earth. The detailed nature of the earliest ecosystems is unknown because no sedimentary rocks older than 3.8 Gyr have been found. Life is unambiguously present at 3.56 Gyr in the Warrawoona Group in Australia; the organisms exhibited wall formation, some kind of motility, phototaxis and mat formation³⁰. This complex and sophisticated set of properties should have required a substantial evolutionary history. In addition, carbon isotopes in the highly metamorphosed sediments of the 3.77-Gyr Isua Group of Greenland indicate that the ratio of organic carbon to carbonate in sediments was similar to the present implying that photosynthetic life was already abundant by Isua time³¹.

Types of ecosystems. Even if individual organisms survive, it is necessary to reestablish a sustainable ecosystem immediately following the impact. The key issue becomes that of the primary producers.

Ecosystems where all the primary productivity is by obligate photosynthetic autotrophs have the primary producers in the top 200 m of the ocean where there is sufficient light. Impacts which sterilized this zone would destroy the global ecosystem and reset biogenesis.

Ecosystems where the primary producers are chemoautotrophs, especially those at hydrothermal vents, would have been much better protected. However, there is still a problem of supplying oxidized material. The present ecosystem at hydrothermal vents ultimately depends on photosynthesis for its oxidants³². A primitive system of that type would die out when oxidants were exhausted. An ecosystem where oxidants are produced inorganically, by oxidation of volcanic SO_2 (ref. 33), or by photolysis of dissolved ferrous iron³⁴, would survive.

A system with photosynthetic primary producers that could act as facultative anaerobic heterotrophs using organic matter and oxidants is also difficult to sterilize. Such reserves are easily exhausted in a well mixed system, such as modern oil spills or the mid-depths of the ancient ocean stirred up by an impact. The oxidant and reductant reservoirs are heterogeneously distributed on the modern Earth, however, and were probably also heterogeneously distributed on the ancient Earth. The structure of the ancient ecosystem would depend on whether sulphates (or sulphites) were present in the ocean.

If significant sulphate (or sulphite) existed, the situation in the global ocean was basically similar to modern closed basins in that excess organic matter existed in the sediments and excess mobile oxidant, the sulphate, existed in the sea water. Heterotrophs could then exist on the interface between the two reservoirs, that is on the sea floor and in very shallow sediments. In the absence of sulphate or sulphite, the main oxidant would be ferric iron, which is less mobile than organic matter and

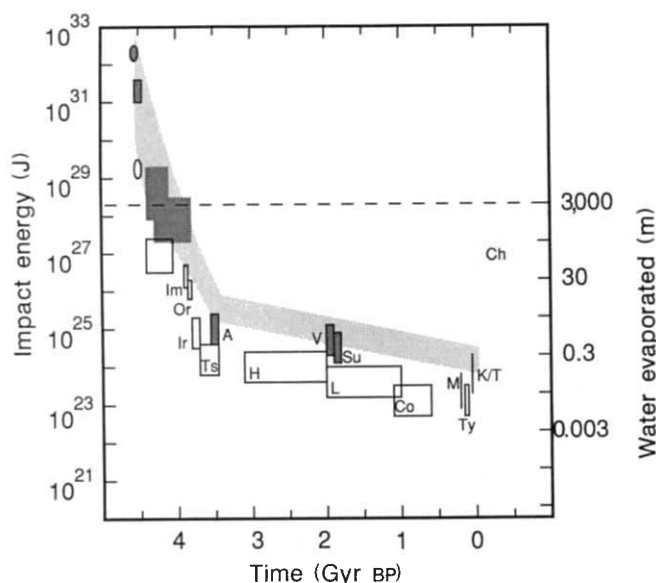


FIG. 1 The largest impacts on Earth and Moon. Open boxes are lunar, filled boxes terrestrial. Lunar craters are Tycho, Copernicus, Langrenus, Hausen, Tsiolkovski, Iridum, Orientale and Imbrium. Terrestrial events are the K/T impact, Manicougan, Sudbury, Vredevort and an impact energy corresponding to the thickness of Archaean spherule beds. Ovals are self energies of formation; the early box refers to a possible Moon-forming impact. Impact estimates between 3.8 and 4.4 Gyr are discussed in the text. The stippled region for Earth is inferred from these data. The depth of ocean vaporized by the impact is also given; the dashed line corresponds to an ocean-vaporizing impact. A possible but extremely unlikely collision with Chiron is placed safely in the future.

tends to sink to the sea floor. Excess ferric oxide would accumulate on the sea floor beneath regions of high organic productivity³⁵. In either case, two reservoirs are not easily exhausted as they are utilized by only a small mass of organisms living at the sea-floor interface.

Additional interfaces within the sedimentary column might escape the surficial heating. The thermal diffusion distance, $\sqrt{\text{diffusivity time}}$, is about a few hundred metres. Deep habitable interfaces, for example, might exist between sedimentary calcium sulphate deposits (or sediments with dissolved sulphate) and sediments with organic matter. By analogy, modern oil-field bacteria exist at the interface of oil and sulphate (or oxygen)-rich water³⁶. Although such deep ecosystems could persist for millions of years, some survivors would need to evolve photosynthesis to sustain life.

Implications. The window in time for the emergence of extant life is defined by a palaeontological considerations on the lower end and by geophysical and planetological considerations on the upper. The necessity for a long evolutionary pre-history to evolve the Warrawoona fossils and the Isua carbon isotopes suggests the existence of life as early as 3.8 Gyr. Here we have been concerned with defining the upper limit. This limit depends on what type of early ecosystem is envisioned and, hence, what one believes is the earliest life form. Did photosynthetic prokary-

otes give rise to organisms that could utilize redox gradients or vice versa? Is it easier for life to originate in surficial environments or the deep ocean? Rather than attempting to resolve these biological issues, we examine the broader implications of our physical inferences.

If one postulates primitive photosynthetic biota as the sole primary producers, then annihilating events in the photic zone probably occurred as late as 3.8 Gyr, and our estimate for the time of the origin of continuous life converges on this date. The situation is less clear if one postulates early deep-marine life. (This situation is also less probable, as no evidence for such organisms exists in either the fossil record or in the evolutionary systematics of advanced life.) In this case, the upper limit may be as great as 4.44 Gyr if the maximum size of impactors was similar to fragmental asteroids or if the few largest objects in a scale-invariant distribution happened to hit the Earth early on. However, size and frequency of impacts should statistically increase as one moves further back in time. Thus, early organisms and ecosystems would have had to be increasingly sophisticated to survive. As this is counter to the usual course of evolution, the upper limit for continuous evolution would be around 4.0 Gyr, with considerable uncertainty. Conversely, a long continuity of life before 3.8 Gyr would imply occupation of the deep sea. □

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Intermediate states in the movement of transfer RNA in the ribosome

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Direct chemical 'footprinting' shows that translocation of transfer RNA occurs in two discrete steps. During the first step, which occurs spontaneously after the formation of the peptide bond, the acceptor end of tRNA moves relative to the large ribosomal subunit resulting in 'hybrid states' of binding. During the second step, which is promoted by elongation factor EF-G, the anticodon end of tRNA, along with the messenger RNA, moves relative to the small ribosomal subunit.

IN its simplest conceptual form, the mechanism of translation¹ must account for three fundamental events: codon-anticodon recognition, peptide-bond formation, and movement of tRNA and mRNA relative to the ribosome. The classical model² proposed that the ribosome contains two binding sites for tRNA, usually called the A (aminoacyl, or acceptor) site and the P (peptidyl, or donor) site. The A site binds the incoming aminoacyl tRNA (delivered to the ribosome as a tRNA-EF-Tu-GTP ternary complex), whose anticodon must read the codon in the mRNA presented in the A site. A peptide bond is then formed between the C-terminal carbonyl group of the nascent polypeptide chain, attached to a P-site-bound tRNA, and the α -amino group of the A-site-bound aminoacyl tRNA. This results in growth, by one amino-acid residue, of the polypeptide

chain, which is then attached to a tRNA in the A site. The empty, or deacylated, tRNA thus remains in the P site. The cycle is completed by transfer of the peptidyl-tRNA to the P site, and release of deacylated tRNA, promoted by elongation factor EF-G and GTP, in a step called translocation.

More recently, several groups³⁻⁶ have presented evidence for the existence of a third site, called the E or exit site, originally proposed by Wettstein and Noll⁷. It is believed that deacylated tRNA binds to the E site before its release from the ribosome. Various rationalizations have been offered for the existence of an E site, although its requirement is not obvious from the point of view of the classical two-site model. A fourth site (referred to as an entry site^{8,9} or recognition site¹⁰) has been proposed as a result of the observation that aminoacyl-tRNA, bound as ternary complex, fails to participate in peptide-bond formation until release of the EF-Tu complex¹¹.

These definitions of the ribosomal binding sites for tRNA emerged from interpreting the results of *in vitro* protein synthesis experiments. Recently, we have searched for structural correlates of these sites, based on increasing evidence that ribosomal RNA is directly involved in interactions with tRNA¹². Using chemical probing methods^{13,14}, we have shown that characteristic sets of bases in rRNA are protected when tRNA is bound to ribosomes. Certain bases in 16S rRNA are protected by tRNA bound to the A and P sites^{15,16}, and in 23S rRNA by tRNA bound to the A, P and E sites¹⁷, as conventionally defined. These site-specific protections are listed in Table 1. Almost all the protected bases are absolutely conserved phylogenetically, and many have been implicated in ribosome function by other biochemical and genetic approaches. The available evidence is consistent with the idea that most of the protected bases are directly involved in binding tRNA to the ribosome. It should be stressed, however, that tRNA-dependent protection could also be the result of induced conformational changes; at present, we cannot distinguish between these two possibilities. Nevertheless, the precise correlation between protection of specific bases in rRNA and occupancy of the ribosomal A, P or E sites allows the use of the protected bases as a diagnostic assay for the location of tRNA in any given state of the translational cycle.

In the experiments described here, we have examined the state of tRNA on the ribosome in the pre- and post-peptidyl transfer stages of the translational cycle in three different kinds of *in vitro* constructs, by direct chemical probing of tRNA-protected bases in rRNA. We found that movement of tRNA occurs independently with respect to the two ribosomal subunits, in two discrete steps. This results in 'hybrid states' of binding in which the acceptor and anticodon ends of a tRNA are in different sites, as defined by the classical model, and could explain why all ribosomes are composed of two dissociable subunits.

Reaction of *N*-acetyl-Phe-tRNA with puromycin

We tested the classical model by examining the binding state of P-site-bound tRNA after peptidyl transferase-catalysed transfer of its acyl group to puromycin¹⁸. The peptidyl-tRNA analogue, *N*-acetyl-Phe-tRNA, is fully puromycin-reactive, forming *N*-acetyl-Phe-puromycin. Before peptide bond formation, *N*-acetyl-Phe-tRNA protected the expected set of P-site-specific bases in 16S and 23S rRNA (Fig. 1*a-f*, lane 2). Unexpectedly, peptide-bond formation between *N*-acetyl-Phe and puromycin abolished the P-site footprint in 23S rRNA, exemplified in Fig. 1*a-c* (lane 3) by the loss of protection of bases G2252, G2253, U2506, U2584 and U2585; the other 23S rRNA P-site protections showed a similar behaviour (data not shown). This was not simply due to loss of the *N*-acetyl-Phe moiety, because deacylated tRNA can also protect these bases when bound to the P site (at high Mg²⁺ concentrations)¹⁷. At the same time, the 16S rRNA P-site footprint persisted after peptide-bond formation (see bases G693, A794, C795 and G926 in Fig. 1*d-f*, lane 3). Another result which is inconsistent with the currently

accepted model was the appearance of the E-site footprint after peptidyl transfer; this was seen in the strong protection of base C2394 (Fig. 1*b*, lane 3) and the weaker, but reproducible protection of bases G2112 and G2116 (Fig. 1*a*, lane 3).

It is clear from the loss of the 23S rRNA P-site footprint that the tRNA must be bound in different 'sites' in the two ribosomal subunits after peptidyl transfer. The simplest interpretation is that the P-site bound tRNA undergoes movement on peptide-bond formation, resulting in a state which resembles E-site binding for 23S rRNA, but in a state closely resembling P-site binding for 16S rRNA. We refer to this as the P/E state. It is remarkable that this movement occurs under these conditions independently of added elongation factors or GTP. Another indication that the binding states differ for *N*-acetyl-Phe-tRNA and the resulting deacylated tRNA comes from the enhanced reactivity of A702 and stronger protection of G1338 in 16S rRNA after peptidyl transfer, a result that was also obtained when deacylated tRNA was bound directly under the same conditions (Fig. 1*e*, lanes 3 and 4).

N-acetyl-Phe-tRNA and the ternary complex

In the previously described experiment, puromycin served as an aminoacyl-tRNA analogue, and the product of the peptidyl transferase reaction was *N*-acetyl-Phe-puromycin, which is released from the ribosome. In the physiological reaction, aminoacyl-tRNA reacts with the peptidyl moiety to yield an extended peptidyl-tRNA that remains bound to the ribosome. To include the participation of aminoacyl-tRNA, and to exclude the possibility that the result observed for the puromycin reaction was atypical, we reacted the Phe-tRNA-EF-Tu-GTP ternary complex with ribosomes containing *N*-acetyl-Phe-tRNA bound to the P site (Fig. 2). This experiment is more technically demanding than the one described in Fig. 1; additional purified components are required, and the ribosomes, tRNAs and factors must be quite active to achieve high overall efficiency in the complete system. The peptidyl transferase reaction falls short of completion in this system, possibly due to the short incubation times required to avoid spontaneous EF-G-independent translocation^{19,22,23}. Nevertheless, the results of this experiment are in close agreement with the results of Fig. 1.

Figure 2 (lane 4) shows the footprints of bound *N*-acetyl-Phe-tRNA; as in the experiment with puromycin, *N*-acetyl-Phe-tRNA protected characteristic P-site residues in both 16S rRNA (Fig. 2*e*, G926) and 23S rRNA (Fig. 2*d*, U2506). On addition of aminoacyl-tRNA ternary complex (Fig. 2, lane 5), significant protection of E-site residues G2112, G2116 (Fig. 2*a*) and C2394 (Fig. 2*c*) was observed, whereas the P-site footprints were maintained on both subunits (Fig. 2*d, e*). By contrast, A-site

TABLE 1 Protection of specific bases in *E. coli* 16S and 23S rRNA by tRNA*

	16S rRNA	23S rRNA
A-site protections		
Strong	G530, A1492, A1493	C2254, A2439, A2451, G2553, ψ 2555, A2602
Weak	A1408, G1494	G1068, G1071, U2609
P-site protections		
Strong	G693, A794, C795, G926, G1401(N7)	G2252, G2253, A2439, A2451, U2506, U2584, U2585
Weak	A532, G1338, A1339, ψ G966	A1916, A1918, U1926, G2505
E-site protections		
Strong	—	ψ C2394
Weak	—	G2112, G2116

* Data are a summary of effects reported in refs 15-17, in which the dependence of these effects on specific structural features of tRNA is described.

residues were protected in 16S rRNA (Fig. 2f, A1492 and A1493), but the 23S rRNA A-site footprint was absent, as shown by the lack of protection of a diagnostic residue C2254 (Fig. 2b) as well as the other 23S A-site bases (data not shown). Thus, after peptidyl transfer, 16S rRNA shows A- and P-site footprints but 23S rRNA shows P- and E-site footprints. Once again, we infer that deacylated tRNA is in the P/E state, whereas the peptidyl-tRNA is simultaneously in the 30S A site and the 50S P site, which we term the A/P state. Further evidence for the A/P state comes from the results of the experiment of Fig. 3. Finally, EF-G-dependent translocation (Fig. 2, lane 6) abolished the 16S rRNA A-site footprint, as shown by the loss of protection of A1492 and A1493 (Fig. 2f). By contrast, the 16S P-site and 23S P- and E-site protections persisted (Fig. 2a, c-e). We infer that the EF-G-dependent translocation event involves movement of peptidyl tRNA (*N*-acetyl-Phe-Phe-tRNA, in this case) with

respect to the 30S subunit, vacating the 30S A site. The result is a transition from the A/P state to the P/P state, in which peptidyl-tRNA is bound in a state equivalent to the classical 'P-site' binding. This requires the deacylated tRNA to vacate the 16S P site, and so it most probably undergoes a shift from the P/E state to the E state, but is not released from the ribosome. EF-G thus catalyses movement of both tRNAs relative to the small subunit; this result is confirmed in the experiment described below, and is in agreement with the observed EF-G-dependent movement of mRNA^{20,21}.

Direct binding to P-site-blocked ribosomes

It was previously found that at high Mg²⁺ concentrations and in the presence of excess deacylated tRNA, peptidyl-tRNA (either (Gly)₃-Phe-tRNA (ref. 24) or *N*-acetyl-Phe-tRNA (refs 25 and 26)) fails to bind to the classical P site, as shown by its unreactivity toward puromycin. After EF-G-dependent translocation, the peptidyl moiety is rendered reactive, indicating that the initial binding of these peptidyl-tRNAs occurs in the A site. Because of the simplicity and efficiency of this system for constructing 'pre-translocation' complexes, in which the state of the ribosome mimics that immediately following peptidyl transfer, it has been used in most studies comparing pre- with post-translocation states. We constructed this type of complex, by first filling the P site with deacylated tRNA and then binding *N*-acetyl-Phe-tRNA, and examining it by chemical probing of rRNA (Fig. 3). In agreement with earlier studies, *N*-acetyl-Phe-tRNA bound in this way was unreactive towards puromycin, and became fully puromycin-reactive after EF-G-dependent translocation.

The binding state of deacylated tRNA resembled that seen in the two previous experiments; the E-site residue C2394 was protected in 23S rRNA (Fig. 3b, lanes 3-5), whereas a strong P-site footprint was observed for 16S rRNA (Fig. 3g, lanes 3-5, and see data presented in ref. 15). Some weaker protection of the 23S rRNA P-site-related residues was also observed, for example at U2506 and U2585 (Fig. 3d, e). We conclude that deacylated tRNA binds primarily in the P/E state under these conditions, and to a lesser degree, in the P/P state. Binding of *N*-acetyl-Phe-tRNA to P-site-blocked ribosomes resulted in strong A-site protections in 16S rRNA (Fig. 3h, lane 4), as predicted from the classical model; at the same time, the P-site footprint of 16S rRNA and the E-site footprint of 23S rRNA persisted (Fig. 3b, g; lane 4). By contrast, little or no protection of the A-site-specific residues C2254, G2553 or ψ 2555 was observed in 23S rRNA (Fig. 3a, c; lane 4); instead, a strong P-site footprint was found (Fig. 3a, d, e; lane 4), in spite of the lack of puromycin reactivity. We conclude that *N*-acetyl-Phe-tRNA bound in this way occupies the A site with respect to 16S rRNA, and the P site with respect to 23S rRNA; in agreement with the results of the previous experiment, peptidyl-tRNA is found in the A/P state in a pre-translocation complex.

On translocation with EF-G and GTP, the 16S rRNA A-site footprint was lost (Fig. 3h, lane 5). By contrast, the P- and E-site footprints of 23S rRNA remained (Fig. 3, lane 5). As for the previous experiment, we conclude that the *N*-acetyl-Phe-tRNA undergoes a transition from the A/P state to the P/P state, as a result of EF-G-dependent translocation. Concomitantly, deacylated tRNA again apparently shifted from the P/E state to the E state (Fig. 3b, lane 5).

The sole difference between the A/P footprint and the standard P-site footprint for 23S rRNA concerns residue A2602. At this residue, tRNA-dependent effects require the presence of a 2',3'-linked acyl group. In previous experiments it was shown that A-site tRNA strongly protects A2602, whereas P-site tRNA causes enhancement of its reactivity. We found that in spite of a strong 23S P-site footprint, A2602 was significantly protected (Fig. 3d, lane 4). On translocation to the P/P state, it became enhanced (Fig. 3d, lane 5). A similar behaviour was found for A2602 in the experiment of Fig. 2 (data not shown). This unusual

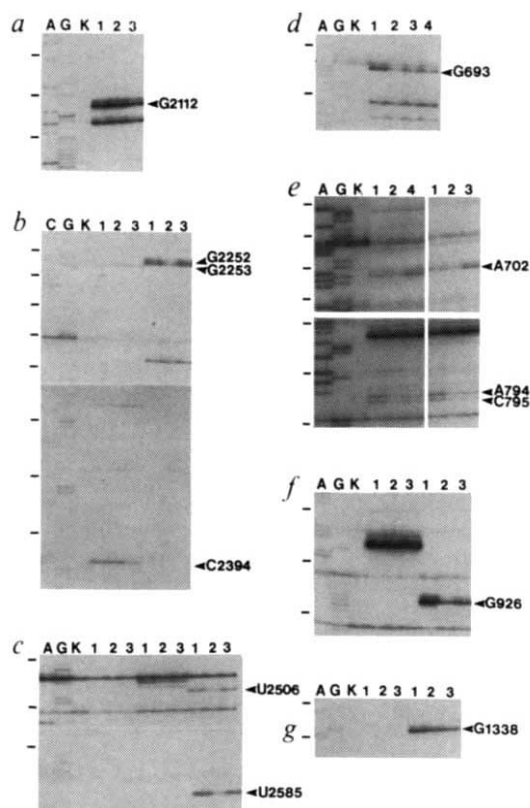


FIG. 1 Protection of bases in rRNA by *N*-acetyl-Phe-tRNA before and after reaction with puromycin. a-c, 23S rRNA; d-h, 16S rRNA. A, C and G are dideoxy chain sequencing lanes and refer to the nucleotide sequence of 16S and 23S rRNA. K, Unmodified control lanes: 1, 70S ribosomes and poly(U); 2, 70S ribosomes, poly(U) and *N*-acetyl-Phe-tRNA (64% bound; 100% puromycin reactive); 3, 70S ribosomes, poly(U), *N*-acetyl-Phe-tRNA and puromycin; 4, 70S ribosomes, poly(U) and deacylated tRNA^{Phe}. a, b (left), c (middle), d and f (right), Results of probing with kethoxal. b (left), c (left), e and f (left), Results of probing with dimethylsulphate. c (right), Probing with CMCT (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate). Positions of chemical attack are determined by primer extension using a set of synthetic primers spaced at 200-250 nucleotide intervals along the entire 16S and 23S rRNA chains^{13,14,17}.

METHODS. Binding was performed by incubating 12 pmol 70S ribosomes with 1 A₂₆₀U poly(U) in 40 μ l of 80 mM potassium cacodylate (pH 7.2), 10 mM MgCl₂, 100 mM NH₄Cl, 1 mM DTT for 5 min at 37 °C. [¹⁴C]-*N*-acetyl-Phe-tRNA (12 pmol; 1,400 pmol per A₂₆₀U) in 20 μ l of the same buffer was then added and incubation continued for 20 min at 37 °C, followed by addition of either 10 mM puromycin in binding buffer (8 μ l) or 8 μ l binding buffer alone, and incubation for an additional 30 min at 25 °C and 8 min at 37 °C. Binding was measured by Millipore filtration³⁹. Puromycin reactivity was tested by incubating 10 μ l of lane 2 complex with 1 μ l 10 mM puromycin on ice for 1 h followed by 5 min at 37 °C, and extraction with ethyl acetate as described¹⁷.

response could reflect a change in the arrangement of the acyl moiety of tRNA resulting from the A/P to P/P transition.

A possible alternative interpretation of the results of the experiment of Fig. 3 is that the P-site footprint seen in lane 4 is attributable to deacylated tRNA. This possibility is excluded by the experiment of Fig. 4. It has previously been shown¹⁷ that protection of several residues in 23S rRNA (G2252, G2253, U2506, U2584 and U2585) by P-site-bound tRNA depends on an intact CCA end in the tRNA. The same effects for the binding of *N*-acetyl-Phe-tRNA were observed in the experiment of Fig. 4 as those seen in the experiment of Fig. 3, even when the P site was first filled by tRNA^{Phe} lacking its 3' terminal CA residues denoted tRNA (-CA). Again, direct binding protected A-site residues in 16S rRNA, and P-site residues in 23S rRNA (Fig. 4, lane 3). The rate of EF-G-dependent translocation is correlated with the intactness of the 3' terminus of tRNA (ref. 27). Accordingly, we used a large excess of EF-G and GTP in this experiment. As in the experiment of Fig. 3, the A-site footprint in 16S rRNA was lost (Fig. 4d, lane 4), and the P-site footprint in 23S rRNA persisted. In contrast to the experiment of Fig. 3, no E-site footprint was observed, in agreement with previous studies which have shown that E-site binding is dependent on deacylated tRNA with an intact CCA terminus^{5,27}.

An interesting difference between the results shown in Figs 3 and 4 is the response of the P-site-specific residues G2252 and G2253 to *N*-acetyl-Phe-tRNA bound in the A/P state. When the P site was blocked with tRNA (-CA), we observed little or no protection of residues G2252 and G2253 by *N*-acetyl-Phe-tRNA (Fig. 4a, lane 3). On translocation (lane 4), these residues were significantly protected. We do not understand the basis of this observation, but it could reflect differences in the state of the A/P tRNA-ribosome complex caused by the binding of

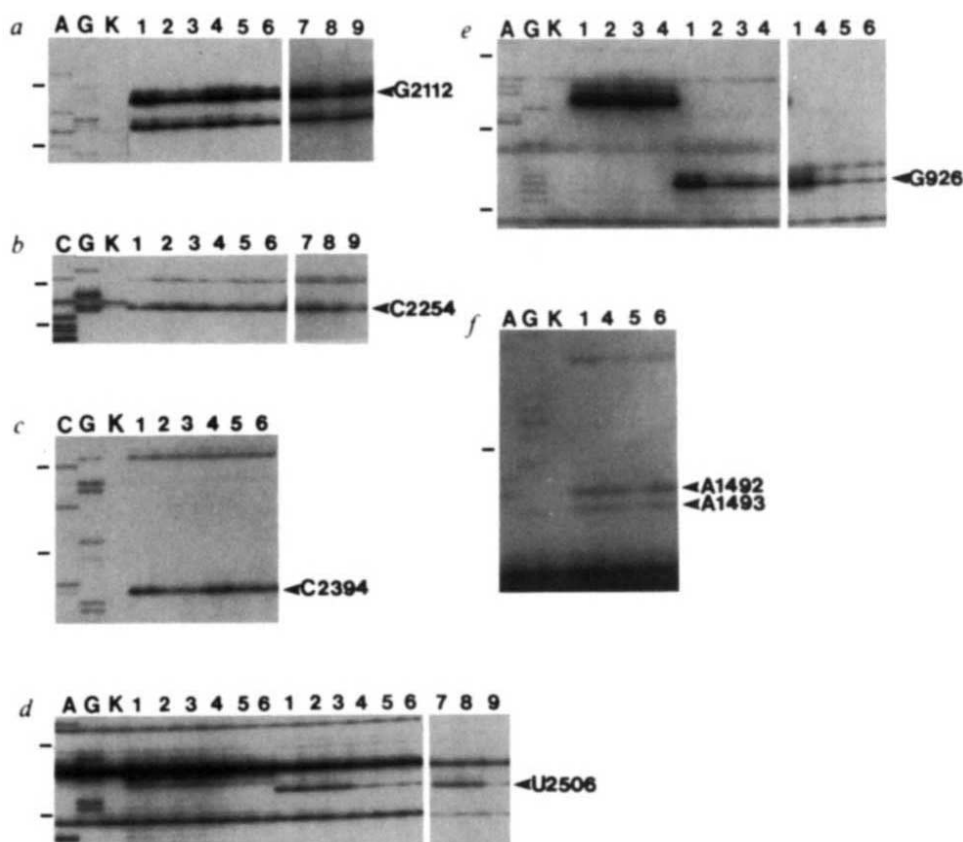
deacylated tRNA in the P/E state. In the experiment of Fig. 4, tRNA (-CA) was unable to bind to the E site, and so must have interacted anomalously, if at all, with 23S rRNA.

Implications for protein synthesis mechanisms

Our findings can be interpreted in terms of a simple model, which is consistent with the results of previous studies on the mechanism of protein synthesis (Fig. 5). The schematic diagram shown in Fig. 5 represents the five known sites of interaction between tRNA and ribosomes—A and P sites on the small subunit, and A, P and E sites on the large subunit (the minimum number of sites required to account for the data available so far). One cycle of elongation is shown, beginning with a ribosome occupied by peptidyl-tRNA, bound to the P site of both subunits, which we term the P/P state. The aminoacyl-tRNA-EF-Tu-GTP ternary complex then joins the translational complex (Fig. 5b) by interaction of its tRNA moiety with the A site of the 30S subunit and its EF-Tu moiety with the 50S subunit in a site involving the α -sarcin loop of 23S rRNA (ref. 28). We call the site of interaction of EF-Tu with the 50S subunit the T site, and define the binding mode of ternary complex as the A/T state. In the A/T state, the aminoacyl end of tRNA is shielded from the peptidyl transferase site¹⁷, and this could be a crucial step in kinetic proof-reading^{29,30} during translation. On the hydrolysis of GTP and the release of EF-Tu-GDP, the aminoacyl tRNA is free to move with respect to the site of the 50S subunit, and is then bound in the A/A state (Fig. 5c). After or during peptide-bond formation, the two tRNAs move with respect to the 50S subunit; the deacylated tRNA binds to the 50S E site, and the newly formed peptidyl tRNA binds to the 50S P site (Fig. 5d). Because their interactions with the 30S subunit are essentially unchanged, the result is a deacylated

FIG. 2 Chemical probing experiments showing the effect of the binding Phe-tRNA-EF-Tu-GTP ternary complex to ribosomes pre-filled with *N*-acetyl-Phe-tRNA on the reactivity of bases in 23S (a-d) and 16S (e, f) rRNA. A, C, G and K are described in Fig. 1 legend. Lanes: 1, 70S ribosomes and poly(U); 2, 70S ribosomes, poly(U) and deacylated tRNA; 3, 70S ribosomes, poly(U), *N*-acetyl-Phe-tRNA and puromycin; 4, 70S ribosomes, poly(U) and *N*-acetyl-Phe-tRNA (65% bound; 91% puromycin-reactive); 5, 70S ribosomes, poly(U), *N*-acetyl-Phe-tRNA, Phe-tRNA, EF-Tu and GTP (80% [¹⁴C]Phe-tRNA bound, as estimated by subtracting the amount of [¹⁴C]-*N*-acetyl-Phe-tRNA bound in lane 4; 16.4% puromycin-reactive calculated relative to [¹⁴C]-*N*-acetyl-Phe-tRNA bound in the complex of lane 4); 6, 70S ribosomes, poly(U), *N*-acetyl-Phe-tRNA, Phe-tRNA, EF-Tu, EF-G and GTP (88% puromycin-reactive); 7, 70S ribosomes and poly(U); 8, 70S ribosomes, poly(U) and deacylated tRNA; 9, 70S ribosomes, poly(U), deacylated tRNA, Phe-tRNA, EF-Tu and GTP (91% [¹⁴C]Phe-tRNA bound).

METHODS. Ribosomes (12 pmol) were incubated with 1 μ M poly(U) in 40 μ l of 80 mM potassium cacodylate (pH 7.2), 8 mM MgCl₂, 100 mM NH₄Cl, 25 mM KCl for 5 min at 37 °C. [¹⁴C]-*N*-acetyl-Phe-tRNA (12 pmol) in 20 μ l of the same buffer was added and incubated for 20 min at 37 °C to allow binding to the P site. To this was added 20 μ l of ternary complex (20 pmol [¹⁴C]Phe-tRNA, 40 pmol EF-Tu-GTP in 80 mM potassium cacodylate, pH 7.2, 8 mM MgCl₂, 100 mM NH₄Cl, 25 mM KCl, 0.5 mM DTT, 3.25 mM GTP, 1 μ M EF-Ts), or puromycin (4 mM in the same buffer), and incubation continued for 1 min at 20 °C. EF-G



was added to a final concentration of 0.8 μ M and incubation continued for 1 min at 37 °C. Puromycin assay was performed as previously described¹⁷ except that the reaction was incubated on ice for 3 h and the short incubation at 37 °C was omitted to avoid spontaneous translocation of *N*-acetyl-Phe₂-tRNA.

FIG. 3 Chemical probing experiments showing protection of bases in 23S (a–f) and 16S (g, h) rRNA in pre- and post-translocation complexes. These complexes were constructed by binding of *N*-acetyl-Phe-tRNA to ribosomes pre-filled with deacylated tRNA without (pre-translocation) or with (post-translocation) addition of EF-G and GTP. Lanes: 1, 70S ribosomes and poly(U); 2, 70S ribosomes, poly(U) and *N*-acetyl-Phe-tRNA (70% bound; 74% puromycin-reactive); 3, 70S ribosomes, poly(U) and deacylated tRNA^{Phe}; 4, 70S ribosomes, poly(U), deacylated tRNA^{Phe} and *N*-acetyl-Phe-tRNA^{Phe} (54% bound; 4% puromycin-reactive); 5, 70S ribosomes, poly(U), deacylated tRNA^{Phe}, *N*-acetyl-Phe-tRNA, EF-G and GTP (56% bound, 104% puromycin-reactive). K, unmodified RNA, C, U, A and G are dideoxy chain sequencing lanes (described in Fig. 1 legend).

METHODS. The P site was blocked by incubating 10 pmol 70S ribosomes with 10 pmol deacylated tRNA^{Phe} and 2 A₂₆₀ U poly(U) in 60 µl of 80 mM K-cacodylate (pH 7.2), 12 mM MgCl₂, 50 mM NH₄Cl, 1 mM DTT for 5 min at 37 °C and 5 min at 20 °C. A-site binding was then performed by addition of 15 pmol [¹⁴C]-*N*-acetyl-Phe-tRNA in 20 µl of the same buffer and incubation for 2 h at 20 °C. As a control, [¹⁴C]-*N*-acetyl-Phe-tRNA was also added to ribosomes in which the P site was not blocked by pre-incubation with deacylated tRNA (lane 2). Translocation was performed by addition of EF-G and GTP to final concentrations of 0.8 µM and 150 µM, respectively, followed by incubation at 37 °C for 10 min.

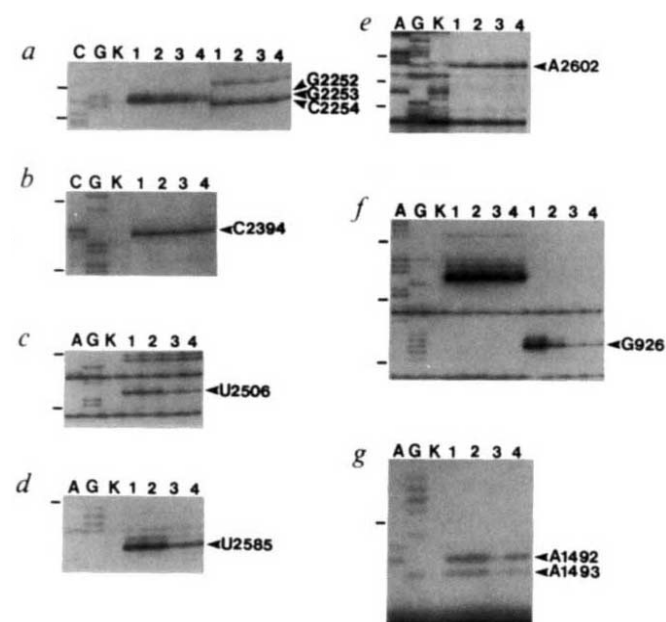
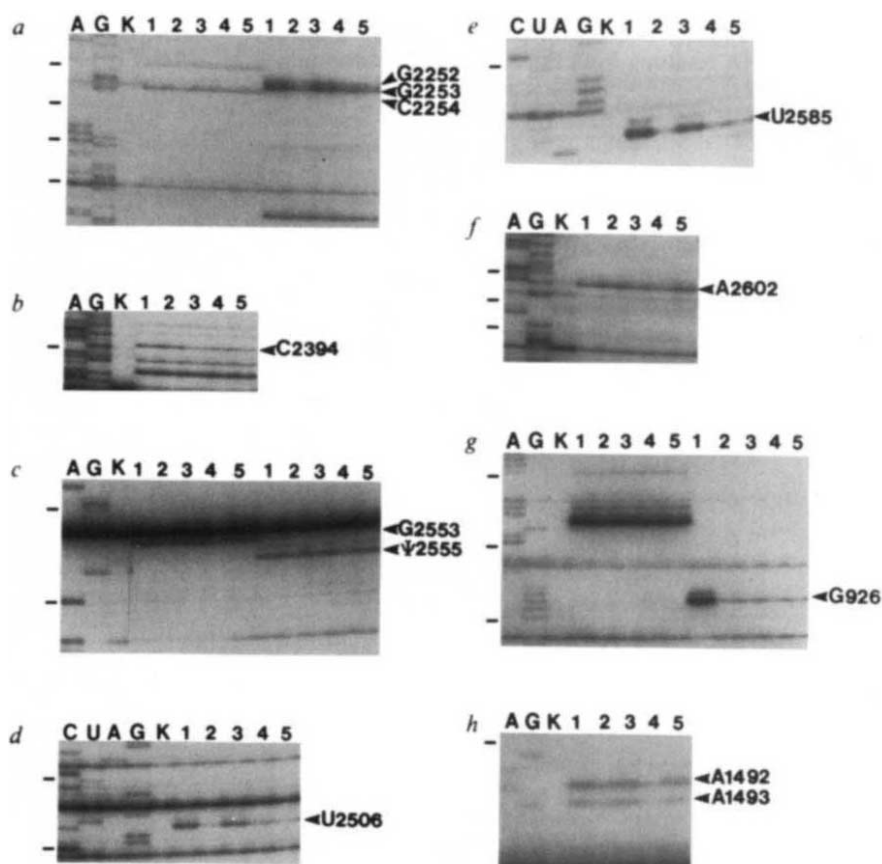


FIG. 4 Protection of bases in 23S (a–e) and 16S (f, g) rRNA caused by binding of *N*-acetyl-Phe-tRNA to ribosomes pre-filled with deacylated tRNA^{Phe} lacking its 3'-terminal CA residues, denoted tRNA^{Phe}(–CA). Lanes: 1, 70S ribosomes and poly(U); 2, 70S ribosomes, poly(U) and tRNA^{Phe}(–CA); 3, 70S ribosomes, poly(U), tRNA^{Phe}(–CA) and *N*-acetyl-Phe-tRNA (62% bound; 6% puromycin-reactive); 4, 70S ribosomes, poly(U), tRNA^{Phe}(–CA), *N*-acetyl-Phe-tRNA, EF-G and GTP (62% bound; 89% puromycin-reactive). A, C, G and K are described in Fig. 1 legend.

METHODS. Binding was performed as described in Fig. 3 legend except that tRNA^{Phe}(–CA) was used instead of deacylated tRNA^{Phe}.

tRNA in the P/E state, and a peptidyl-tRNA in the A/P state. One implication of this result is that the peptidyl moiety itself remains in a fixed position relative to the structure of the ribosome. In our *in vitro* experiments this movement of tRNA with respect to the large ribosomal subunit occurs independently of added factors or GTP. This step represents a fundamental departure from the classical model for protein synthesis. Finally, realignment of the two tRNAs with respect to the 30S subunit is accomplished in a step requiring EF-G and GTP (Fig. 5e). This results in movement of peptidyl-tRNA to the P/P state, and deacylated tRNA from the P/E to the E state.

The most remarkable observation is that movement of tRNA occurs independently on the two ribosomal subunits. This implies that a tRNA bound to a given binding site on the small subunit can interact with more than one binding site on the large subunit, and vice versa. Thus a tRNA bound to the P site of the small subunit can interact with either the P or E sites on the large subunit, corresponding to the P/P or P/E states, respectively.

We conclude that there are at least six distinguishable binding states for tRNA: A/T, A/A, A/P, P/P, P/E and E (Fig. 5). The newly observed states have previously escaped detection mainly because of the inherent limitations of traditional methods. Binding site models have usually been based on the results of filter binding and puromycin reactivity. Experiments of this kind can establish a minimum for the number of binding sites, and whether or not a given tRNA is puromycin-reactive, but cannot distinguish, for example, whether there is more than one puromycin-unreactive state. Binding states of deacylated tRNA are also indistinguishable by the puromycin criterion. By redefining the operational definition for classical A- and P-site binding in terms of ribosomal RNA footprints, we have substituted direct structural correlates for the traditional experimental criteria, which has permitted us to examine the state of

tRNA binding in a completely independent way. An example of the limitation of the traditional criteria is the previous failure to detect the A/P state. On the basis of puromycin reactivity, the A/P and A/A states are indistinguishable. For reasons not yet understood, tRNA in the A/P state is not puromycin-reactive, although it yields a 23S rRNA footprint which closely resembles that of tRNA bound in the puromycin-reactive P/P state. An indication that the tRNA-23S rRNA interactions differ between the A/P and P/P states comes from the reactivity of A2602, which is weakly protected in the A/P state but significantly enhanced in the P/P state (Table 1; Fig. 3f, lanes 2, 4 and 5).

We should point out that our interpretation is based on the assumption that our previous assignment of protected bases to occupancy of specific ribosomal sites holds for the *in vitro* complexes that we studied. It is possible that conformational changes involving certain components of the translational machinery could result in protection of these same bases, and thereby mimic the occupancy of a specific ribosomal site by tRNA. We think that such possibilities are unlikely. For example, an A-site-bound tRNA could cause protection of the same bases that are protected by P-site tRNA. But such an A-site tRNA would, at the same time, have to fail to protect the A-site bases, and cause protection of the E-site residues. In any event, such an explanation cannot account for the loss of P-site protections and the appearance of E-site protections after the peptidyl transferase reaction that we observed (Fig. 1).

Fluorescence spectroscopy provides a direct physical method for observing the state of ribosome-bound tRNA. Fluorescent probes are sensitive to local changes in solvent polarity and molecular anisotropy, and can also be used to measure inter- and intramolecular distances by nonradiative energy transfer. Hardesty and co-workers, using this approach, have observed changes in fluorescence quantum yield and fluorescence anisotropy of fluorescent probes attached to tRNA, after peptide bond formation^{31,32}. Their results show that at least some part of the tRNA moves relative to ribosomal proteins S21 and L11. They have proposed that the tRNA, rather than the nascent

peptide, moves during the peptidyl transferase reaction. In contrast to the hybrid site model, they envisage that movement of mRNA is also accomplished by the movement of tRNA generated by the peptidyl transferase reaction. We would interpret their results in terms of a manifestation of the P/P to P/E transition.

The molecular basis of tRNA movement

By the very nature of the translation process, movement of mRNA and tRNA relative to the ribosome is required. Here we have shown that this movement occurs in two discrete steps. Our results indicate the occurrence of molecular rearrangements beyond simple relocation of tRNA molecules. When tRNA is bound to the 16S rRNA A site, it can interact at the same time with either the A or P sites of 23S rRNA; tRNA bound to the 16S rRNA P site can interact with either the P or E sites of 23S rRNA. If the interaction of the anticodon stem-loop moiety of tRNA with 16S rRNA remains essentially the same, irrespective of the state of the interaction of the tRNA with the large subunit, as implied by our footprinting evidence, then one or more of the macromolecular components (tRNA or ribosome) must undergo some kind of structural rearrangement. For example, tRNA may flex or bend, as has often been suggested (see refs 33, 34, and refs cited therein), or some feature of one of the ribosomal subunits, such as the head or platform of the small subunit or the L7/L12 stalk of the large subunit may move³⁵, or the mode of interaction between the two ribosomal subunits may change³⁶. Although we cannot exclude the participation of any of these classes of molecular movement, we argue that some sort of relative movement of the 30S and 50S subunits is implicit in the model of Fig. 5.

An important feature of our model is that tRNA is always anchored at one end during movement. In the shift to the hybrid state (Fig. 5c, d), the tRNAs are anchored on the small subunit while shifting with respect to the large subunit. During the EF-G-dependent event (Fig. 5d, e), they are anchored to the large subunit. It is probably significant that each tRNA always

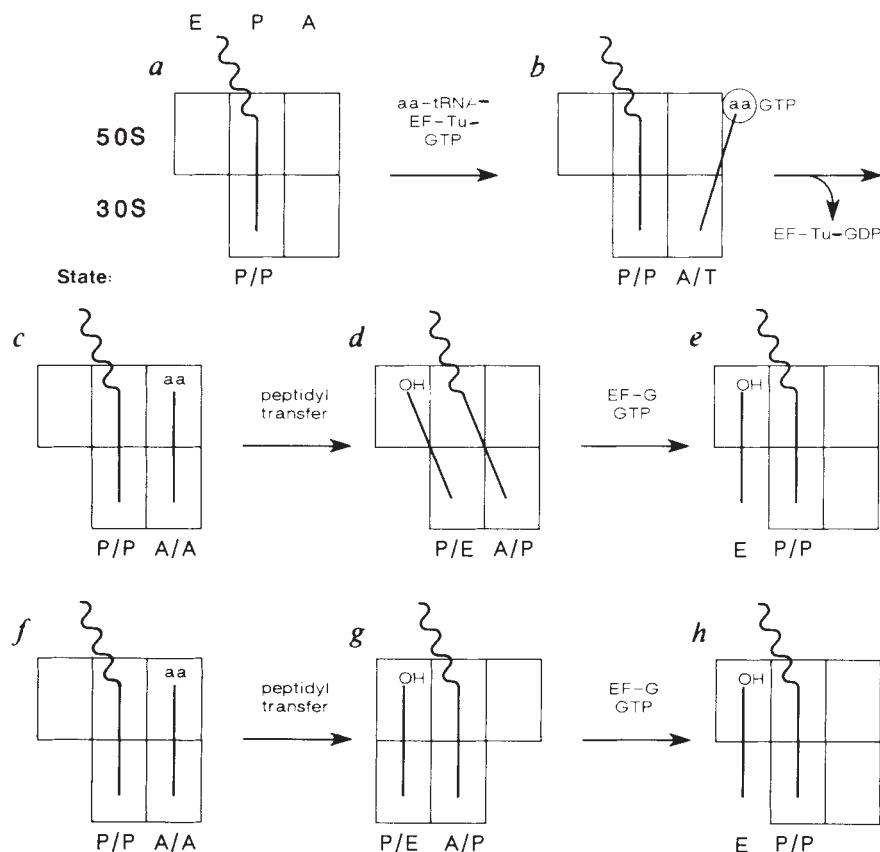


FIG. 5 A scheme showing the binding states of tRNA on the ribosome through a single cycle of translational elongation. The 50S and 30S subunits are symbolized by the upper and lower rectangles; the 50S is divided into E, P and A sites, and the 30S into P and A sites, based on the lack of evidence for a 30S E-site component. —, tRNA; ~~~, nascent polypeptide chain; aa, aminoacyl group; circle, EF-Tu. The binding states shown at the bottom describe the 30S and 50S sites occupied by a tRNA at each step. *a*, Ribosome with a peptidyl-tRNA in the P/P state, equivalent to classical P-site binding. *b*, An aminoacyl-tRNA-EF-Tu-GTP ternary complex binds in the A/T state, making A-site contacts on the 30S subunit; the 50S subunit interacts with EF-Tu, and is shielded from tRNA. *c*, EF-Tu-GDP is released after hydrolysis of GTP, allowing interaction of aminoacyl-tRNA with the 50S A site. *d*, On peptide-bond formation, both tRNAs move relative to the 50S subunit, from P/P to P/E and A/A to A/P. *e*, EF-G promotes movement of the tRNAs relative to the 30S subunit, from the P/E to the E state and the A/P to the P/P state. For further details, see text. An alternative representation of steps *c*-*e* is shown in *f*-*h*. Here, the *c* to *d* step and the *d* to *e* step rearrangements are shown as involving movement of the 30S subunit relative to the 50S subunit in steps *f* to *g* and *g* to *h*, respectively. The latter scheme is similar to one proposed earlier by Bretscher³⁶.

interacts with both ribosomal subunits in each binding state (except, perhaps, in the E state)—the anticodon stem-loop region with the small subunit, and the CCA end with the large subunit.

An alternative representation of the last two steps of our model is shown in Fig. 5*f-h*, formally equivalent to Fig. 5*c-e*, but in this case represented as a reversible shift in the spatial relationship between the two subunits, rather than movement of tRNA relative to a static ribosome. This form of the model stems from, and emphasizes, the fact that tRNA movement occurs in two steps, each of which is characterized by changes in the tRNA-dependent footprint involving the rRNA for only one of the subunits. The idea that tRNA movement is somehow coupled to relative movement of the two ribosomal subunits was first proposed by Bretscher³⁷ and Spirin³⁶ to account for the two-subunit structure that is found in all ribosomes. Spirin's model also predicted the spontaneous movement of the CCA moiety of tRNA from the 50S A site to the P site during the peptidyl transferase step. But he envisioned movement of tRNA as a whole, relative to the 50S subunit, as an EF-G-dependent step. Bretscher suggested two alternative ways in which the two subunits could move relative to one another to produce hybrid tRNA binding sites, one of which is similar to that shown in Fig. 5*f-h*.

Several lines of evidence indicate a relationship between subunit-subunit interaction and tRNA binding. Most intriguing is the behaviour of residue A702 of 16S rRNA, which is reactive in 30S subunits, and partially protected by subunit association (D. M. and J. McWhirter, unpublished results). Binding of tRNA in the P/P state results in a small additional protection, whereas tRNA in the P/E state causes significant enhancement (Fig. 1*e*). This result indicates that a change in some aspect of the mode of subunit interaction accompanies rearrangement to hybrid binding states. Residues A1418 and A1483 in 16S rRNA are partially protected by subunit association, and their protection is strengthened by tRNA binding; no tRNA-dependent protection is found in the absence of 50S subunits. The additional protection of these bases is not due simply to tRNA-stimulated

subunit-subunit interaction, because other 50S-protected residues are unaffected by tRNA binding under the same conditions. A third line of evidence is that the reactivity towards chemical probes of a group of bases, which we term class III sites, is affected by tRNA binding (independent of 50S subunits), subunit association and the binding of certain antibiotics³⁸. Finally, we note that bases whose reactivity responds to tRNA binding are often located close to bases that are affected by subunit association.

If the two-step model for translocation is based on two different states of subunit-subunit interaction (Fig. 5*f-h*), a strong prediction is that the binding of two tRNAs to the ribosome excludes the binding of only one tRNA in a hybrid state. This would provide a simple explanation for the observation that when deacylated tRNA is bound alone, it is in the P/E state, but on adding aminoacyl-tRNA to the complex, it apparently shifts to the P/P state^{16,17}. Because aminoacyl tRNA can bind only in the A/A state, we argue that its binding requires rearrangement of the 30S-50S interaction, and both tRNAs must therefore adopt a nonhybrid binding state. There is some experimental evidence for such a mechanism, obtained by using the reactivity of A702 in 16S rRNA as a marker for the hybrid state, as suggested above. When deacylated tRNA is bound alone, A702 is reactive; when aminoacyl-tRNA is then bound in addition, the reactivity of A702 is diminished, as predicted¹⁶. By contrast, binding of *N*-acetyl-Phe-tRNA, which binds in the A/P state under similar conditions, fails to result in protection of A702.

Finally, we suggest that the driving force for movement of tRNA into the hybrid state depends on the different affinities of the sites on the large subunit for the acceptor ends of tRNA species that are generated after peptide-bond formation. Immediately after peptidyl transfer, there is a deacylated tRNA in the P site and a peptidyl tRNA in the A site; the higher affinities of these tRNA species for the E and P sites of the 50S subunit, thermodynamically favour the P/P to P/E and the A/A to A/P transitions, respectively. This ensures that the movement mechanism is unidirectional, from sites A to P to E. □

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Rescue of *bicoid* mutant *Drosophila* embryos by Bicoid fusion proteins containing heterologous activating sequences

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The maternal gene *bicoid* (*bcd*) determines pattern in the anterior half of the *Drosophila* embryo. It is reported here that the injection of *bcd*⁻ mutant embryos with messenger RNAs that encode proteins consisting of heterologous acidic transcriptional activating sequences fused to the DNA-binding portion of the *bcd* gene product, can completely restore the anterior pattern of the embryo.

THE *bcd* gene organizes development in the anterior half (head and thorax) of the *Drosophila* embryo¹. The molecular basis for this morphogen function is a concentration gradient of Bcd protein spanning the anterior two thirds of the syncytial blastoderm stage embryo². The Bcd protein is synthesized from *bcd* mRNA that is localized at the anterior tip of the embryo^{3,4}, and the concentration of Bcd protein determines positional values along the antero-posterior axis⁵. Genetic analyses indicate that the zygotic expression of *hunchback* (*hb*), a gap gene that is expressed early in a distinct domain in the anterior 45% of the embryo, is regulated by the Bcd protein⁶⁻⁹. Recent experiments demonstrate that Bcd protein binds specifically to the *hb* promoter region and induces the expression of *hb* in both *Drosophila* Schneider cells and the embryo¹⁰. The binding of Bcd protein to multiple copies of Bcd-binding sites in the upstream region of fusion genes is sufficient to generate an *hb*-like expression pattern in the early embryo¹¹.

The N-terminal portion of the 489-amino-acid Bcd protein⁴ contains a homeodomain (residues 92-151), a protein sequence which is conserved among many gene products participating in embryonic pattern formation¹²⁻¹⁴, and which is believed to direct specific DNA binding¹⁵⁻¹⁸. Recent experiments indicate that at least several of the homeodomain proteins involved in *Drosophila* embryonic pattern formation are transcriptional regulatory proteins¹⁹⁻²¹. Near the N-terminal end of the Bcd protein there is a repeat that is found in the *paired* gene product³; this repeat is a sequence of ~30 amino acids, rich in histidine and proline, which is also present in other gene products in *Drosophila*. The function of this repeat, however, is unknown. The C-terminal part of the Bcd protein contains an acidic region (residues 347-414, with 16 acidic and 5 basic residues) as well as a glutamine-rich region (M- or opa-repeat; residues 256-289)¹². The Bcd protein is also rich in hydroxyl amino acids (37 serines, 27 threonines and 19 tyrosines). In *Drosophila* Schneider cells, and most probably in the embryo, the Bcd protein is phosphorylated at several sites¹⁰. Wild-type Bcd protein, as well as certain other homeodomain-containing proteins in *Drosophila*, activate transcription in yeast^{18,22}, and the protein product of several mutant *bcd* alleles have been analysed for their transcriptional function in yeast²².

Recent studies on yeast transcriptional activators indicate that a typical activator contains two separable functions: one that directs the activator to specific DNA sequences and another that interacts with some component of the transcriptional

machinery²³⁻²⁶. For example, the yeast transcriptional activators GAL4 and GCN4, which are involved in galactose metabolism and amino-acid synthesis, respectively, contain acidic activating sequences that interact with the transcriptional machinery^{27,28}. The activating sequences of GAL4 can be functionally replaced either by acidic peptides encoded by *Escherichia coli* DNA, or by an acidic peptide designed to form an amphiphilic α -helix^{29,30}. Not all acidic peptides function as activating sequences; some aspect of structure, perhaps an α -helix, is required³⁰. GAL4, or its derivatives that contain one or more acidic activating sequences fused to the DNA-binding portion of GAL4, also stimulate gene expression in mammalian, insect and plant cells, provided that the sequence to which GAL4 binds is inserted near the promoter of the test gene³¹⁻³⁴. Other classes of activating sequences have been described^{35,36}; none of these, to our knowledge, has been shown to function in many different eukaryotic cells as do the acidic sequences, and some of them have been shown to work only in restricted cell types³⁶.

Here we describe experiments that show that the Bcd protein activates transcription from the Bcd-binding sites in three different systems—yeast cells, *Drosophila* Schneider cells, and *Drosophila* embryos. We also report that the N-terminal half of the Bcd protein (residues 1-246), which contains the DNA-binding function, has little transcriptional activation function in all three systems. When tested in *Drosophila* embryos by injecting the corresponding mRNA, residues 1-246 of Bcd only partially rescues the mutant *bcd*⁻ phenotype, whereas fusion proteins consisting of residues 1-246 of Bcd attached to heterologous acidic activating sequences fully rescue the *bcd*⁻ phenotype.

Transcriptional activation by Bcd derivatives

Transcriptional activity in yeast was measured in cells bearing an integrated *GAL1-lacZ* fusion gene³⁷ containing three strong Bcd-binding sites^{10,11}. These yeast cells also contained plasmids expressing, from the yeast *ADHI* promoter³⁸, genes encoding the *Drosophila* Bcd protein or one of its C-terminal deletion derivatives. The activities of Bcd derivatives in *Drosophila* Schneider cells were measured by co-transfecting the cells with a reporter plasmid containing the gene for chloramphenicol acetyl transferase (CAT) fused to the *hb* gene, and effector plasmids expressing the Bcd protein or one of its derivatives. For the assay in embryos, mRNAs encoding the Bcd protein or one of its derivatives were synthesized *in vitro*. These were then co-microinjected with a plasmid containing the reporter gene *hb-298CAT* into the anterior half of pre-polecell stage embryos from females homozygous for *bcd*^{E1}, a strong *bcd* allele bearing a large deletion^{1,4,22}. The injected mRNA is efficiently translated, as revealed by immunohistological stainings of the injected embryos for Bcd protein (data not shown). The reporter DNA might enter the nuclei during the rapid syncytial nuclear divisions and the *hb-CAT* fusion gene is transcribed under the control of the Bcd protein¹⁰. About 3 h after injection, at the onset of gastrulation, CAT enzyme activity was measured in extracts prepared from the injected embryos (Table 1).

TABLE 1 Activities of Bcd protein and its deletion derivatives

Effector	Yeast β -gal activities (hb-GAL1-lacZ)	<i>Drosophila</i> Schneider cells CAT activities (hb-298CAT)	<i>Drosophila</i> embryos CAT activities (hb-298CAT)	Rescue of <i>bcd</i> ⁻ mutant phenotype
Wt Bcd(1-489)	125	45	40	complete
Bcd(1-396)	12	36	35	complete
Bcd(1-348)	12	9*	19	complete
Bcd(1-246)	13	8	8	partial
Bcd(1-111)	<1	2	2	none
None	<1	2	2	—

The stimulatory effect of Bcd protein on *hb* transcription was assayed in yeast, *Drosophila* Schneider cells and *Drosophila* embryos. Yeast—plasmids expressing Bcd or its deletion derivatives were transformed into a yeast strain (GGY1:MA630R) containing an integrated *GAL1-lacZ* fusion gene; the *hb* promoter element bearing the Bcd protein binding sites¹⁰ was located upstream of the *GAL1* gene. *Drosophila* Schneider cells—the effector plasmids, which express from the *Drosophila* metallothionein promoter various Bcd deletion derivatives, were transfected into Schneider cells together with the reporter plasmid hbCAT-289 which expresses the *E. coli* CAT gene from the *hb* promoter bearing the Bcd-protein-binding sites. CAT activities (per cent acetylated forms of total chloramphenicol) are shown.* In Schneider cells, instead of Bcd(1-348), the derivative Bcd(1-338) was used. *Drosophila* embryos—in *vitro* transcribed mRNAs encoding Bcd protein and its deletion derivatives were co-injected with the reporter plasmid hbCAT-298 into the anterior half of pre-pole cell stage embryos derived from females homozygous for *bcd*^{E1}. CAT activities were determined at the onset of gastrulation. The rescue of the *bcd*⁻ mutant phenotype is described in detail in Fig. 2 and Table 3a.

The yeast strain used in these experiments is derived from GGY1(*ura3*, *leu2*, *his3*, Δ *gal4*, Δ *gal80*) (ref. 46) by integrating at the *URA3* locus a modified *GAL1-lacZ* fusion gene. The plasmid pMA630R, which was used to generate GGY1:MA630R, was constructed by inserting the *HindIII-MluI* fragment from the plasmid hbCAT-298 (ref. 10) into the *XhoI* site of LR1 Δ 1 Δ 2 μ (ref. 37). The plasmids expressing Bcd deletion proteins were constructed in two steps. First, the *XbaI* linker (5'-CTAGTCTAGACTAG-3'), which contains translational stop codons in all three reading frames, was inserted at different positions in the *bcd* gene in the plasmid pTN3bcd, an expression vector (Sp6 promoter) for the Bcd-encoding region with the frog globin mRNA leader³⁹. Then, either the intact or modified Bcd encoding regions were inserted into the *HindIII* site of the yeast plasmid AAH5 containing the yeast *ADHI* promoter³⁸. The *XbaI* linker was inserted at the following positions: *PstI* site (at residue 111), *Sall* site (at residue 246), *AccI* site (at residue 348) and *BglII* site (at residues 396). The β -galactosidase (β -gal) activities were measured in yeast cells grown in synthetic minimal medium lacking leucine with glycerol and ethanol as carbon sources⁴⁷. For Schneider cells, modified Bcd encoding regions containing stop codons at various positions were inserted into plasmid pRmHa-3, a *Drosophila* expression vector bearing the metallothionein promoter⁴⁸. Unlike the Bcd(1-348) protein, which was generated by inserting the stop codon linker at the *AccI* site, we used Bcd(1-338) in Schneider cells, the stop linker being inserted at the *Aval* site. The procedures for Schneider-cell transfection, the reporter plasmid hbCAT-298 and CAT assay were as described previously^{10,49}. Twelve hours after transfection, the culture medium was replaced by the medium containing 1 mM CuCl₂ to induce the metallothionein promoter, and the CAT activities were assayed 24 h later. The transfection efficiency was normalized by the β -gal activity expressed from the third transfected plasmid pAcLacZ (ref. 10). For the assays in yeast and Schneider cells, synthesis of the various Bcd proteins was tested by immunoblotting analysis (Fig. 1). For *Drosophila* embryos, mRNAs coding for Bcd protein or its deletion derivatives were transcribed *in vitro* by Sp6 RNA polymerase⁵⁰ from plasmid pTN3bcd and its derivatives (see above). The mRNAs (0.5 μ g μ l⁻¹) were co-injected with the reporter plasmid phbCAT-298 (1 μ g μ l⁻¹) into the anterior half of pre-pole cell stage embryos from females homozygous for *bcd*^{E1}. At the onset of gastrulation, extracts were prepared and CAT activities were determined as described previously¹⁰. All assays described above were done in quadruplicate. For assays in yeast and Schneider cells, the standard error of the mean (s.e.m.) was normally <20%; for assays in *Drosophila* embryos the s.e.m. was 20% (for wild-type (wt) Bcd), 26% (for Bcd(1-396)) and 50–60% (for the shorter truncations).

Table 1 describes transcriptional activation by the Bcd protein and various deletion derivatives in the three assays, as well as the results of experiments measuring rescue of *bcd*⁻ embryos (see later). Wild-type Bcd protein activates transcription in all three systems. Deletion of 83 C-terminal residues from the Bcd protein creates a derivative containing residues 1–396, Bcd(1-396), which activates transcription only 10% as efficiently in yeast as does the wild-type Bcd protein, but 80% as efficiently as does the wild-type protein in *Drosophila* cells and embryos. Derivative Bcd(1-338) has lost most of its ability to activate transcription in Schneider cells, but activates ~50% as efficiently as wild-type Bcd protein in *Drosophila* embryos. Derivative Bcd(1-246) activates transcription only slightly in all three systems, and Bcd(1-111), which contains only a third of the homoeodomain, is inactive. Figure 1 shows that proteins of the expected size are synthesized in yeast and Schneider cells. However, the results are obscured somewhat by our lack of knowledge of protein levels in the three test systems but, when taken with the results of the following section, indicate a correlation between the ability of a Bcd derivative to activate transcription in the embryo and its biological function as a morphogen. The fragment Bcd(1-396) has lost part of the acidic sequence of the Bcd protein, and Bcd(1-348) has lost all of it; thus this acidic sequence is not required for all of the transcriptional activation function of the Bcd protein in the embryo.

Morphogenetic activities

We tested the morphogenetic activity of the Bcd deletion derivatives by injecting small amounts of corresponding *in vitro*-synthesized mRNAs into the anterior tip of embryos obtained from *bcd*^{E1} mutant females and analysing the cuticle phenotype after one day of development (Tables 1 and 3a; Fig. 2). Embryos not injected do not develop heads and thoraces, but instead

form duplications of posterior structures anteriorly (Fig. 2a). Depending on the concentration, injection of mRNA coding for wild-type Bcd protein can completely rescue the mutant phenotype, and larvae frequently hatch, giving rise to adult flies³⁹. Of the deletion derivatives, both Bcd(1-396) and Bcd(1-348) are also able to rescue the mutant phenotype completely. By contrast, Bcd(1-111) does not rescue any aspect of the *bcd*⁻ mutant phenotype. The derivative Bcd(1-246) only partially restores the wild-type pattern: it suppresses the formation of posterior structures at the anterior, and induces with high frequency thoracic structures, and more rarely, structures of the segmented region of the head, the labial, maxillary or mandibular segment. But even when the mRNA coding for the derivative Bcd(1-246) is injected at high concentration (5 μ g μ l⁻¹; Table 3a), the embryos never hatch (see Fig. 2d). Embryos injected with mRNA encoding the fragment Bcd(1-246) resemble those from females that are homozygous for two of the weak *bcd* alleles, *bcd*^{E5} and *bcd*¹¹¹ (ref. 1), which are caused by amber mutations at amino acids 259 and 257, respectively²². Thus our data indicate that the C-terminal third of the protein, containing the acidic region (residues 347–414), is not essential for the ability of the protein to rescue the mutant phenotype. Our results also indicate that only those forms of Bcd that activate transcription in the embryo—wild type, Bcd(1-396) and Bcd(1-348)—can completely rescue *bcd*⁻ embryos. We now show that Bcd(1-246), a derivative that activates transcription poorly in all three systems, can rescue *bcd*⁻ mutant embryos when fused to heterologous acidic activating sequences.

Bcd fusion proteins

We attached Bcd(1-246) fragments to (1) the acidic activating sequences of the herpes viral activator VP16 (residues 411–

491)^{40,41}; (2) the acidic activating region II of GAL4 (residues 768–881)²⁸; (3) part of region II of GAL4 (residues 851–881)²⁸; or (4) the *E. coli*-derived acidic activating sequences B6, B17, and B42 (ref. 29) (Fig. 1). As expected, each of these fusion proteins activates transcription in yeast cells more efficiently than does the N-terminal fragment of Bcd alone (Table 2).

We tested the morphogenetic activity of the Bcd fusion proteins by injecting the respective mRNAs synthesized *in vitro* into the anterior tip of embryos obtained from mutant females homozygous for *bcd*^{Et}. Three of these mRNAs, at appropriate concentrations, can completely rescue the *bcd*^{Et} mutant phenotype (Table 3b). The phenotypes obtained are described in detail in the legend to Fig. 2 and are discussed below. A complete rescue of head and thorax was obtained when the embryos were injected with mRNAs encoding fusion proteins of fragment Bcd(1–246) attached to either GAL4(851–881) or the *E. coli* DNA-derived activating sequences B6 and B17. Up to 19%, 37% and 20% of the developing embryos were rescued, respectively, by each of these activators. In each case, as is the case when wild-type *bcd* mRNA was injected, the frequency of hatching larvae was ~30% of the number of embryos with morphologically normal heads.

With the exception of the three potent activators described below, the phenotypes obtained by injecting mRNAs encoding the various fusion proteins and deletion derivatives can, depending on the concentration, result in embryos with phenotypes mimicking those of various *bcd* alleles. These phenotypes include that of wild-type flies (complete rescue; class 5 in Table 3, and Fig. 2e,f), the phenotype of weak alleles (partial rescue; class 3 and Fig. 2d), and the phenotype of strong alleles (no rescue); class 2 and Fig. 2a). In addition, a novel phenotype is observed in embryos that are injected with these mRNAs at high concentrations. In some cases, abdominal segmentation is distorted (seen in embryo of classes 3, 4 and 5), whereas in severe cases, in addition to defective abdominal development, the head anlagen are apparently shifted so far towards the posterior that head involution cannot occur, and embryos are generated bearing a hole in the cuticle anterior to the thorax (class 4). The latter phenotype can be explained by a Bcd protein gradient with a much higher overall concentration throughout the embryo than that of the wild-type Bcd protein gradient. As Bcd protein determines positions along the antero-posterior

axis in a concentration-dependent manner⁵, overall higher concentrations of Bcd would result in a shift of the blastoderm anlagen towards the posterior.

The injection of mRNAs that encode potent Bcd fusion proteins bearing VP16, GAL4(768–881) or B42 cause another novel phenotype (class 1, see Fig. 2b). In these embryos, neither anterior nor posterior structures form anteriorly, but development is suppressed, resulting in embryos with a partial abdominal pattern and an open anterior end. By injecting the latter two mRNAs at lower concentrations, at least a partial rescue is obtained, whereas the most potent activator, the Bcd(1–246)–VP16 fusion protein, has a deleterious effect on the anterior development even when its mRNA is injected at a very low concentration (Table 3b). These three potent Bcd fusion

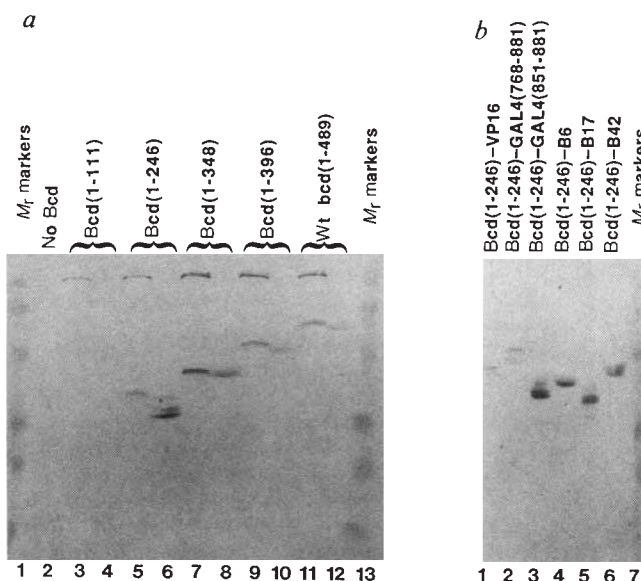


FIG. 1 Immunoblotting assays. *a*, The intact Bcd protein and deletion derivatives synthesized in yeast (lanes 4, 6, 8, 10 and 12; lane 2: yeast extracts without Bcd protein) and *Drosophila* Schneider cells (lanes 3, 5, 7, 9 and 11). The predicted relative molecular masses (M_r) are ($M_r \times 10^{-3}$): wild-type Bcd, 54 (ref. 4); Bcd(1–396), 44; Bcd(1–348), 39; Bcd(1–246), 28; and Bcd(1–111), 13. The monoclonal antibody does not react with Bcd(1–111). The apparent M_r of the proteins including wild-type Bcd protein are higher than predicted. The electrophoretic mobilities of the proteins synthesized in yeast are relatively higher than those of the proteins from Schneider cells; the possible effect of different post-translational modifications in yeast and Schneider cells is discussed in the text. *b*, The Bcd fusion proteins synthesized in yeast. The predicted M_r of the fusion proteins are ($M_r \times 10^{-3}$): Bcd(1–246)–VP16 (326 amino acids), 36; Bcd(1–246)–GAL4(768–881) (361 amino acids), 40; Bcd(1–246)–GAL4(851–881) (278 amino acids), 31; Bcd(1–246)–B6 (334 amino acids), 37; Bcd(1–246)–B17 (278 amino acids), 31; and Bcd(1–246)–B42 (337 amino acids), 37. Apparently, the fusion protein Bcd(1–246)–B6 synthesized in yeast migrates faster than expected, and it is possible that most of this protein is degraded; this result may be related to the observation that Bcd(1–246)–B6 is not very active in yeast cells (see Table 2). The sizes of M_r markers (from BioRad) are ($M_r \times 10^{-3}$): 130, 75, 50, 39, 27 and 17.

METHODS. Procedures of preparing proteins from yeast and Schneider cells were as previously described^{10,51}. For the yeast proteins (*a*), the yeast cells were grown in 10 ml synthetic minimal medium (lacking leucine) with glycerol and ethanol (optical density at 600 nm (OD_{600}) at 0.8–1.1), and for Bcd fusion proteins in *b*, the yeast cells were grown in 5 ml synthetic minimal medium with glucose (OD_{600} at 1.0–1.7). The amount of protein samples loaded on the protein gel was normalized by the density of the yeast cultures (OD_{600}). Proteins from Schneider cells were expressed from the *Drosophila* metallothionein promoter as described in the legend to Table 1. The derivative Bcd(1–348) in Schneider cells should be Bcd(1–338) (see legend to Table 1 for details). A 12% SDS gel was used to separate the proteins. The first antibody (mouse anti-Bcd)² was diluted 1:1,000 and the second antibody (alkaline phosphatase conjugated rabbit anti-mouse IgG) was diluted 1:1,500. Staining procedure was according to the manufacturer's instructions (BRL).

TABLE 2 Activities of Bcd fusion proteins

Effector	Yeast β -gal activities (hb-GAL1-lacZ)	<i>Drosophila</i> embryos Rescue of <i>bcd</i> ^{Et} mutant phenotype
Wt Bcd	125	complete
Bcd(1–246)	13	partial
Bcd(1–246)–VP16	1,307	lethal
Bcd(1–246)–GAL4(768–881)	953	lethal
Bcd(1–246)–GAL4(851–881)	342	complete
Bcd(1–246)–B6	43	complete
Bcd(1–246)–B17	105	complete
Bcd(1–246)–B42	367	lethal
None	<1	–

Yeast—plasmids expressing Bcd fusion proteins were transformed into a yeast strain, and β -gal activities measured. See legend to Table 1 for further details. *Drosophila* embryos—the rescue of the *bcd*^{Et} mutant phenotype by injection of *in vitro* transcribed mRNAs coding for Bcd protein derivatives is described in detail in Fig. 2 and Table 3b. Various activating sequences were fused to the *bcd* gene in the plasmid pTN3bcd; these plasmids were also used to synthesize the mRNAs for the assays in *Drosophila* embryos. The activating sequences were obtained from the HindIII–SalI fragment of the plasmid p58dTX for VP16, and the EcoRI–SalI fragments for all the others^{28,29}. Then, these *bcd* fusion genes were inserted into the HindIII site of the yeast expression vector AAH5 (ref. 38). The yeast strain used was GGY1:MA630R. The assays were done in quadruplicate and the standard error was normally <20%.

proteins could cause the toxic effects by: (1) activating the Bcd-protein target gene(s) to some intolerable level; (2) activating some otherwise non-target genes because of single Bcd-binding sites that may be randomly distributed in the *Drosophila* genome; or (3) having the general inhibitory effect on gene expression called 'squenching' (refs 42, 43).

Discussion

Here we have described experiments in which we assayed the morphogenetic properties of Bcd and various Bcd derivatives by injecting mRNAs into embryos derived from *bcd*⁻ mutant females. We have also described the transcriptional activating properties of Bcd and various Bcd derivatives in yeast, *Drosophila* Schneider cells and *Drosophila* embryos. Our results with the Bcd(1-246) derivative are particularly indicative. This fragment, which contains the DNA-binding homeodomain, has a weak transcriptional activation function as assayed in all systems, compared with wild-type Bcd protein, and can only

partially rescue *bcd*⁻ mutant embryos. However, when fused to certain acidic activating sequences—peptides known to confer transcriptional activation in many eukaryotes—Bcd(1-246) fully rescues *bcd*⁻ mutant embryos.

Our results with Bcd deletion derivatives also indicate a correlation between the ability of a given *bcd* allele to activate transcription as assayed in the embryo, and its ability to rescue the *bcd*⁻ mutant phenotype. Thus, for example, of the derivatives tested, three (wild type, Bcd(1-396) and Bcd(1-348)) rescued the wild-type phenotype, and these derivatives stimulated transcription most efficiently in the embryo. We note that two of these proteins (Bcd(1-396) and Bcd(1-348)) are severely impaired in the activation function as assayed in yeast. These results are consistent with the idea that the fragments Bcd(1-396) and Bcd(1-348) are modified in the embryo, perhaps by phosphorylation, to potentiate or to acquire an activating function. It is also possible that, for example, the sequence of residues 247-348, which is rich in Gln, functions as an activating sequence

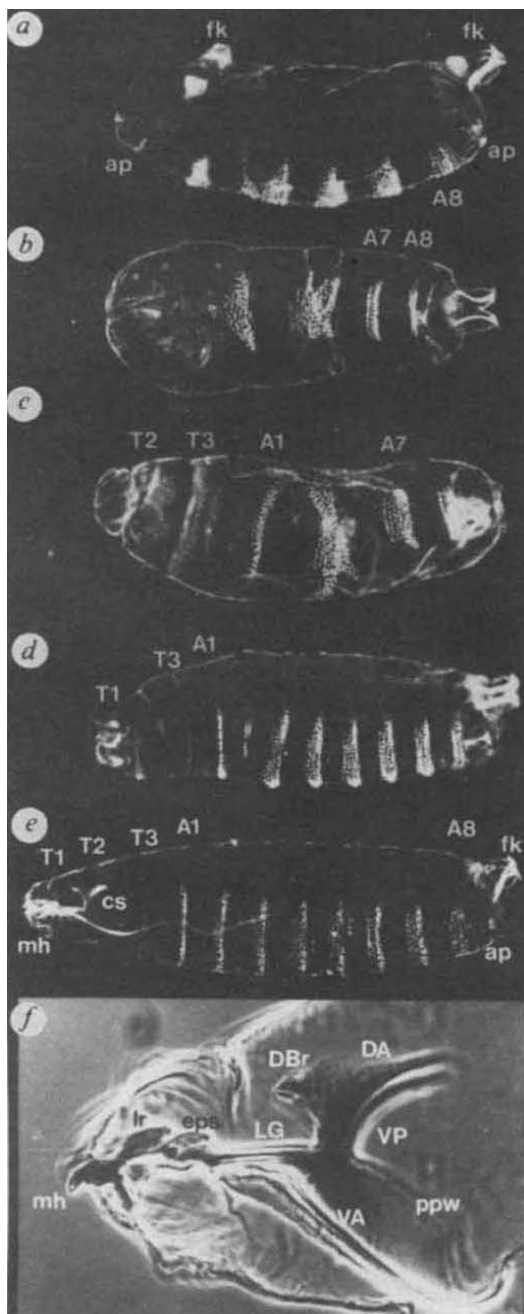


FIG. 2 Rescue of *bcd*⁻ mutant *Drosophila* embryos by Bcd-acid-sequence fusion proteins. Shown are cuticular preparations of *bcd*⁻ embryos injected with *in vitro* synthesized mRNAs encoding Bcd derivatives. The phenotypes of these embryos are grouped into five classes (also see legend to Table 3a, b). *a*, Strong *bcd*⁻ phenotype (class 2 in Table 3a, b). Heads and thoraces of the embryos are replaced by a duplication of posterior telson structures (for example, filzkörper, and anal plates)¹. All embryos containing filzkörper structures anteriorly are classified as *bcd*⁻ mutant, including a few embryos that form thoracic structures as well as filzkörper anteriorly. *b*, Anterior structures not developed (class 1). Neither anterior nor posterior structures develop at the anterior of the embryo, and the anterior-most identifiable structures are the third or fourth abdominal denticle belt. Usually the cuticles have a large hole at the anterior end, though in some cases (as shown) the anterior end is closed. *c*, Partial rescue with open anterior ends (class 4). The embryos develop, with a high variability, one to three thoracic denticle bands, an antennal sense organ, a maxillary sense organ, and/or mouth hooks, but they never develop a complete head skeleton. The cuticles have a hole of varying size at the anterior. Frequently some of the abdominal segments are fused or deleted. Embryos of this class form headfolds shifted towards the posterior of the embryos. Thus, the abdominal anlagen are obviously compressed and the segmentation distorted, whereas the head region is vastly expanded and can no longer be involuted—a possible cause for the hole at the anterior end. *d*, Partial rescue (class 3). The phenotype of this class resembles those of weak *bcd*⁻ mutants (for example, *bcd*⁵⁵). Thoracic and gnathal structures but no structures derived from the anterior-most 20% of the blastoderm fate map, for example, the labrum, are formed. *e*, Complete rescue (class 5). Such embryos develop complete head and thorax including the anterior-most structure, the labrum. In a few cases the proportioning of the head skeleton deviates slightly from the wild-type form, and distortions of the abdominal segmentation appear frequently. After rescue by either wild-type Bcd protein or Bcd fusion proteins, 30–50% of the larvae in this class hatch, and some develop into adult flies. *f*, Higher magnification of the head region of a rescued *bcd*⁻ mutant embryo. All the structures present in wild-type embryos can be identified here.

METHODS. See legends to Table 1 and Table 2 for a description of the expression vectors used in our study. The mRNAs, synthesized *in vitro* by Sp6 RNA polymerase⁵⁰, were extracted with phenol-chloroform and chloroform, precipitated with ethanol, washed with 70% ethanol, and diluted in diethyl-pyrocyanate-treated water to different concentrations. The mRNAs obtained were tested in an *in vitro* wheat-germ translation extract (Amersham) in the presence of [³⁵S]methionine. Measurement of ³⁵S incorporation into the proteins showed that all the mRNAs were of similar quality, and SDS-PAGE revealed proteins of the expected *M_r* (data not shown). The mRNAs were then injected into the anterior tips of the pre-pole-cell stage embryos obtained from homozygous mutant *bcd*^{E1} females according to standard procedures¹. Embryos developed for 40 h at 18 °C and the cuticles were prepared as previously described¹. The anterior is always at the left and dorsal at top when lateral views are shown (*a*, *d*–*f*); *b* and *c* show ventral views. The mRNAs injected were (in $\mu\text{g } \mu\text{l}^{-1}$): Bcd(1-246)–GAL4(851–881), 0.1 (*a*); Bcd(1-246)–VP16, 1.2 (*b*); Bcd(1-246)–GAL4(768–881), 1.2 (*c*); Bcd(1-396), 0.8 (*d*); Bcd(1-246)–B17, 0.4 (*e*); Bcd(1-246)–B6, 0.4 (*f*). A1–A8, Ventral abdominal denticle bands; ap, anal plates; cs, cephalopharyngeal skeleton; DBr, dorsal bridge; DA, dorsal arm; eps, epis-tomal sclerite; fk, filzkörper; LG, lateralgräte; lr, labrum; mh, mouth hook; ppw, wall of pharynx posterior to ventral arm; T1–T3, thoracic denticle bands; VA, ventral arm; VP, ventral plate⁵².

TABLE 3a Rescue of *bcd*⁻ embryos by Bcd and its deletion derivatives

RNA concentration ($\mu\text{g } \mu\text{l}^{-1}$)		Bcd(1-111)				Bcd(1-246)					Bcd(1-348)				Bcd(1-396)				Wild-type Bcd			
		1.20	0.40	0.10	0.02	5.00	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02
Phenotype	<i>n</i>	31	16	34	21	33	56	62	60	63	65	47	56	39	84	61	59	55	58	71	54	65
1. Anterior not developed		3	6	0	0	48	38	15	5	0	25	15	14	8	25	18	5	4	26	7	13	2
2. <i>bcd</i> ⁻ mutant		97	94	100	100	3	9	58	93	100	0	4	27	85	1	18	88	96	2	9	41	97
3. Partial rescue		0	0	0	0	9	5	18	2	0	28	38	52	8	35	36	7	0	21	32	37	2
4. Partial rescue (anterior open)		0	0	0	0	40	48	7	0	0	29	19	7	0	20	21	0	0	29	18	9	0
5. Head and thorax completely rescued		0	0	0	0	0	0	0	0	0	18	23	0	0	19	7	0	0	22	35	0	0

TABLE 3b Rescue of *bcd*⁻ embryos by Bcd and Bcd fusion proteins

RNA concentration ($\mu\text{g } \mu\text{l}^{-1}$)	Bcd(1-246)-VP16				Bcd(1-246)- GAL4(768-881)				Bcd(1-246)- GAL4(851-881)				Bcd(1-246)-B6				Bcd(1-246)-B17				Bcd(1-246)-B42			
	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02	1.20	0.40	0.10	0.02
	n=19	59	52	33	24	73	64	43	46	34	73	51	29	57	64	38	25	45	72	54	40	35	56	67
Phenotype																								
1. Anterior not developed	95	92	96	55	58	18	28	14	13	3	21	2	10	5	23	5	12	0	18	30	63	12	60	28
2. <i>bcd</i> ⁻ mutant	5	7	4	45	0	5	13	65	2	26	10	98	7	2	17	95	0	2	11	20	5	14	5	34
3. Partial rescue	0	0	0	0	0	60	56	21	6	29	38	0	7	32	30	0	20	40	50	50	5	43	20	37
4. Partial rescue (anterior open)	0	0	0	0	42	15	3	0	59	29	22	0	60	25	28	0	64	35	11	0	27	31	14	0
5. Head and thorax completely rescued	0	0	0	0	0	0	0	0	19	12	10	0	17	37	3	0	4	20	11	0	0	0	0	0

Shown are the results of micro-injections of mRNAs encoding different Bcd derivatives into embryos that were obtained from homozygous *bcd*^{E1} females¹. The cuticles of the developed embryos were analysed and the phenotypes classified (on the left of each Table) as described in Fig. 2. The mRNAs encoding various Bcd derivatives are shown at the top of each Table; concentrations as indicated. *n*, Number of cuticles analysed that developed from 100 injected embryos. The phenotypic classes are specified as a percentage of total embryos analysed. See legend to Fig. 2 for details of experiments. To test the statistical significance of the data obtained, several series of injections were repeated and the average difference between two injection series were <50%; different phenotypic classes were never obtained.

in the embryo, but not in Schneider cells or in yeast.

The results of the mRNA injection experiments also allowed us to begin to analyse the effects of activating strengths and concentrations of the Bcd protein derivatives on embryonic development.

First, the concentration of the activators can vary within a certain range to allow normal embryonic development. For example, the Bcd fusion proteins bearing GAL4(851-881), B6 or B17 fragments can rescue the *bcd*⁻ mutant phenotype when their mRNAs are injected at concentrations differing by a factor of >10 (Table 3b), although injection of mRNAs at high concentration tends to distort abdominal development. This observation is consistent with previous genetic data showing that the embryos from females bearing as many as six copies of the *bcd* gene show normal development, but when there are eight copies of the *bcd* gene in the female, the abdominal segmentation frequently is abnormal and the embryos may die (refs 4 and 5; T. Berleth and W.D., unpublished results). The 'posteriorward' shift of position on the fate map with increased concentrations seems to be largely independent of the activator strength, because the weak activator Bcd(1-246) also shows this effect (data not shown).

Second, the activities of the Bcd derivatives can also vary within a certain range, and still allow the development of normal larvae. For example, both Bcd(1-348) and wild-type Bcd protein can completely rescue the *bcd*⁻ mutant phenotype, although their transcriptional stimulatory activities, as determined in *Drosophila* embryos, differ by a factor of two (Table 1).

Our experiments demonstrating that Bcd protein activates transcription in yeast cells from the *hb* upstream element further support the idea that one function of Bcd protein during development is to stimulate the expression of *hb* gene by directly binding to the sites located upstream of the *hb* gene promoter. In addition to activating the target gene(s), the maternal Bcd protein could have other functions during early development of *Drosophila* embryos. For example, Bcd protein could inhibit

the translation of the maternal *caudal* gene mRNA (refs 2 and 44), and Bcd protein would be required to define the domain of expression of another gap gene (*Krüppel*) independent of *hb* (ref. 45). Our results indicate that if these effects of Bcd protein are direct and if they are essential to the development of *Drosophila* embryos, they must be specified by the N-terminal portion (residues 1-246) of Bcd which is present in the Bcd fusion proteins that are able to rescue the *bcd*⁻ mutant phenotype. □

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Movement of microtubules by single kinesin molecules

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Kinesin is a motor protein that uses energy derived from ATP hydrolysis to move organelles along microtubules. Using a new technique for measuring the movement produced *in vitro* by individual kinesin molecules, it is shown that a single kinesin molecule can move a microtubule for several micrometres. New information about the mechanism of force generation by kinesin is presented.

MYOSIN, dynein and kinesin are motor proteins which share the ability to convert chemical energy derived from the hydrolysis of ATP into mechanical work. This chemomechanical transduction process involves a cyclic reaction of a motor molecule with a cytoskeletal polymer; an actin filament in the case of myosin and a microtubule in the instances of dynein and kinesin. After binding to the filament, the motor protein is thought to undergo a conformational change, the power stroke, that produces an increment of movement. The protein then releases the filament before rebinding at another site along the filament and thus initiating another cycle. Changes in the affinity of the motor protein for the polymer and the timing of the power stroke are believed to be controlled by transitions between the intermediate states in the ATPase cycle^{1,2}.

To elucidate the molecular events underlying chemomechanical transduction, one must determine the force produced by an individual molecule, the distance through which a filament is displaced on ATP hydrolysis and the transition rates between successive mechanical and biochemical states. Several factors conspire, however, to obscure these molecular details. For example, it is difficult to extrapolate results to the molecular level when dealing with muscle fibres that possess as many as 10^9 myosin molecules, or with cilia that contain in excess of 10^4 dynein molecules. There are also many different cytoplasmic proteins that modify or regulate the actions of the motor proteins themselves. Motility assays that involve either coating a small bead and observing its movement along a filament^{3,4}, or coating a surface with a purified motor protein and observing the translocation of a complementary purified filament^{5,6}, have circumvented some of these difficulties. Although it is now possible to perform measurements of force⁷ and displacement⁴ with such *in vitro* assays, interpretation of the results at the molecular level is precarious owing to uncertainties about the numbers and possible interactions of the motor molecules involved.

We report here the development of an *in vitro* assay in which purified bovine brain kinesin is adsorbed at low density to the glass surface of an experimental chamber and the ATP-dependent movement of individual microtubules is observed by dark-field microscopy. The stoichiometry of the reaction indicates that a microtubule can be translocated for several micrometres by one kinesin molecule, a tetramer^{8,9} comprising two light chains (relative molecular mass 62,000 (62K)) and two identical heavy (120 K) chains that terminate in globular, ATP- and microtubule-binding heads^{10–14}. By measuring the speed of movement of microtubules driven by known numbers of kinesin molecules, we can delimit several properties of kinesin's cyclic reaction with a microtubule, including the time of attachment between a kinesin molecule and a microtubule, the force against which one kinesin molecule can work and the magnitude of the displacement produced on hydrolysis of one ATP molecule.

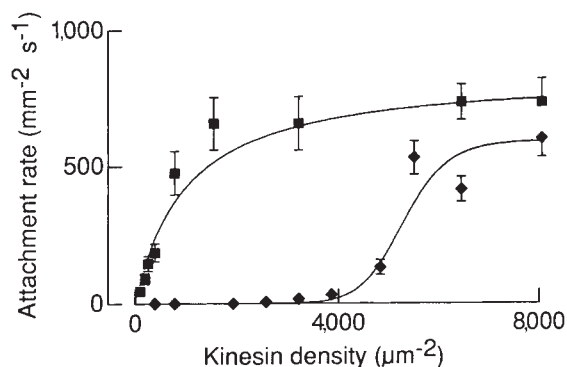
Microtubules move at low kinesin density

To determine the number of kinesin molecules required to move a microtubule, we decreased the density of kinesin adsorbed to successive glass surfaces by exposing them to solutions containing kinesin at progressively lower concentrations. In this way we reduced the number of motor molecules that could potentially interact with the microtubules suspended in a solution that was subsequently introduced into the chamber. If only a single kinesin molecule were required, movement would be expected at very low kinesin densities. To the contrary, movement occurred only when the kinesin density exceeded a relatively high threshold value (Fig. 1); kinesin adsorbed directly onto the glass surface of the chamber, from a solution containing a concentration of 100 nM, resulted in the attachment of numerous microtubules which then moved along the glass surface, but microtubules failed to adhere to a surface treated with kinesin at a concentration of 50 nM. Like kinesin¹⁵, both myosin¹⁶ and dynein¹⁷ must exceed a minimum density before supporting filament movement *in vitro*. Because virtually all of the kinesin molecules in such solutions are adsorbed onto a glass surface within the incubation time of 120 s, this threshold for movement corresponds to a kinesin density of $2,000\text{--}4,000\text{ }\mu\text{m}^{-2}$ (see Fig. 1 legend for the calculation).

The density threshold could be interpreted to indicate that the movement of a microtubule is a highly cooperative event that requires several kinesin molecules. An alternative explanation is that, when kinesin molecules adsorb to glass, most denature on the surface and are unable to produce movement. Only when the surface is nearly covered might the last few kinesin molecules adsorb in a configuration that can sustain

FIG. 1 The rate of attachment of microtubules to a kinesin-coated surface. When kinesin is adsorbed directly onto a glass surface (◆), a threshold density is necessary for microtubules to attach and move. On the other hand, if the glass surface is first pretreated with tubulin and cytochrome *c*, a threshold is no longer apparent and attachment and movement occur at much lower densities (■).

METHODS. The kinesin was prepared at 70–90% purity from bovine brain²³ and its concentration, estimated by gel densitometry with a bovine-serum-albumin standard²³, refers to the tetramer composed of equimolar heavy and light subunits^{8,9}. Tubulin of concentration 50 μM , prepared from bovine brain²⁹, was polymerized in the presence of 4 mM MgCl_2 , 1 mM GTP, and 10% dimethyl sulphoxide. In 100 μM ATP, the ATP turnover rate per kinesin molecule increased from 0.06 s^{-1} to 1.8 s^{-1} on addition of 7 μM polymerized, dimeric tubulin. This microtubule-stimulated ATPase activity is comparable to that reported by others^{22,23}. Experimental solutions were introduced into a chamber, of dimensions 18 mm $\times$ 4.5 mm $\times$ ~110 μm , composed of a coverslip and a microscope slide (Gold Seal, Clay Adams) separated by greased slivers of coverslip glass. For one curve (◆), the chamber was first filled for 120 s with standard buffer solution (80 mM PIPES, 1 mM EGTA, 2 mM MgCl_2 , adjusted to pH 6.85 with KOH) into which kinesin had been diluted. Control experiments showed that kinesin adsorbs to the surface of the chamber with a half life of 20–30 s, which is roughly the time necessary for a kinesin molecule to diffuse through the depth of the chamber. Before being infused into the chamber, microtubules were diluted ~100-fold to a final concentration of 0.5 μM into the standard buffer solution augmented with 5 μM taxol and 1 mM ATP. The continuous curve is a plot of the equation $(600 \text{ mm}^{-2} \text{ s}^{-1})\rho^{1/2}/(1 + \rho^{1/2})$, in which ρ is the kinesin density (see below) divided by 5,300 μm^{-2} . For the other curve (■), as well as for the experiments shown in the following figures, the chamber was first filled for 120 s with the undiluted tubulin solution to pretreat the surface, then filled for 120 s with the kinesin-containing standard buffer solution to which 4 μM cytochrome *c* (Sigma) had been added. The solution into which the microtubules had been diluted was also augmented with 4 μM cytochrome *c* prior to infusion into the chamber. The continuous curve is a plot of the Langmuir isotherm $(850 \text{ mm}^{-2} \text{ s}^{-1})\rho/(1 + \rho)$, in which ρ is the kinesin density divided by 1,000 μm^{-2} . The density of kinesin on the glass surface was calculated from the concentration of kinesin, the chamber's volume (~9 μl), and the area of the glass surface (160 mm^2) with the assumption that all the kinesin adsorbed. We confirmed that at least 80% of the kinesin adsorbs within



120 s by introducing the kinesin-depleted solution from one experimental chamber into a second pretreated chamber and measuring the rate of microtubule attachment to the glass surface. The geometry of the chamber minimized the gradient of protein adsorbed to the glass surface when solutions were introduced from one side of the chamber: the chamber's ~100 μm depth permitted the infusion of solutions within ~1 s, a time much shorter than the time required for diffusion to the surface. By measuring the microtubule attachment rate across the chamber we confirmed that over the central 2 mm of the chamber, the region from which the reported data were derived, the density variation was less than 15%. The slide surface of the chamber was viewed in an inverted microscope (IM-35, Zeiss) with darkfield optics (condensor lens, numerical aperture 1.2–1.33, Olympus; 50 $\times$ objective lens, numerical aperture 0.5–0.85, Nikon) with illumination by visible light from a 100 W mercury-arc lamp (HBO 100W/2, Osram). Images of microtubule movements were projected through a 20 $\times$ ocular onto a silicon-intensified-target camera (C2400-8, Hamamatsu). The attachment of microtubules was measured by sequentially observing several fields of view (~25 $\mu\text{m} \times$ ~35 μm) for times ranging from one to several minutes and counting the number of microtubules that attached to and began to move across the surface. The attachment rate was this number divided by the observation time and the viewing area. Experiments were performed at room temperature (20–23 $^{\circ}\text{C}$).

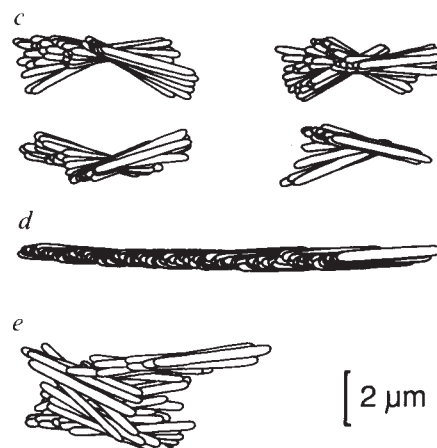
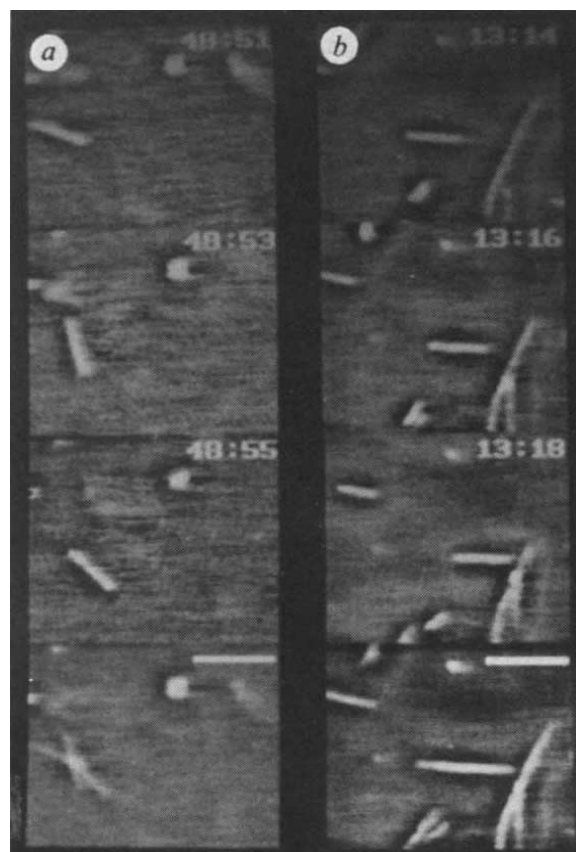


FIG. 2 Photographed video images (*a* and *b*) and tracings of images (*c*, *d* and *e*) of microtubules moving across surfaces coated with kinesin. The three upper panels of (*a*) or (*b*) depict the positions of microtubules at 2 s intervals; the lowest panel shows a superposition of the three images. The tracings present successive video images at 133 ms intervals. In each of the examples in *a* and *c*, for which the kinesin density was 24 μm^{-2} , the microtubule pivoted about a nodal point where a kinesin molecule was presumably located. In three of the traced instances, the microtubule moved until its trailing end nearly reached this nodal point before leaving the surface; the fourth dissociated precociously. The ATP concentrations ranged from 30 μM to 1 mM. At a kinesin density of 2,400 μm^{-2} (*b* and *d*) and an ATP concentration of 100 μM , microtubules moved smoothly and with negligible torsion. A detached microtubule undergoing Brownian motion near the glass surface (*e*) produced an erratic pattern of movement quite distinct from that in the other panels.

movement. This would explain why the critical density corresponds to an almost complete surface coating by the 80 nm long, 5–15 nm diameter kinesin molecules^{11,13,18} and why long microtubules, which would be expected to interact with a larger number of kinesin molecules, require the same critical density as short microtubules.

To test this hypothesis, we attempted to prevent denaturation of kinesin by pretreating the glass surface with other proteins before adsorbing kinesin. Pretreatment with haemoglobin (15 μM), dimeric tubulin (10 μM), or cytochrome *c* (4 μM) enabled microtubules to move at densities less than one-third of those required with untreated glass. After the most successful pretreatment, exposing the surface to tubulin (50 μM) followed by cytochrome *c* (4 μM) and kinesin, the movement of microtubules occurred at kinesin densities as low as $\sim 2 \mu\text{m}^{-2}$. The threshold behaviour was no longer observed and the number of moving microtubules decreased gradually as the kinesin density was reduced (Fig. 1).

The behaviour of individual microtubules moving at low kinesin density on a pretreated glass microscope slide suggested that few motor molecules were participating. Each moving microtubule rotated erratically about a roughly vertical axis through a fixed point on the surface (Fig. 2*a, c*), presumably as the result of thermal forces, or of torques produced when a kinesin molecule bound to different protofilaments. When its trailing end reached this nodal point, the microtubule dissociated from the surface and diffused back into solution. By contrast, microtubules exhibited negligible rotation when moving across a surface coated with kinesin at a high density, presumably because the microtubules were restrained by numerous kinesin molecules attached along their lengths (Fig. 2*b, d*). Both patterns of movement were distinct from the brownian motion of free microtubules (Fig. 2*e*).

One kinesin molecule moves a microtubule

At low kinesin densities, microtubules thus appear to be attached at only one point, where we infer that at least one kinesin molecule is situated. To determine how many molecules lie at such a point and thus the stoichiometry of the reaction between kinesin molecules and microtubules, we measured the rate at which microtubules attached to and moved across the surface as a function of kinesin density. When the surface was treated with tubulin and cytochrome *c*, the microtubule attachment rate was directly proportional to the kinesin density (Fig. 3*a*); the slope of unity in the double-logarithmic plot is consistent with the hypothesis that the movement reaction requires only one kinesin molecule. Had motion required the cooperation of two or more kinesin molecules, the association rate would have fallen more abruptly as the kinesin density was reduced.

The rate at which microtubules left the surface, the detachment rate, also accorded with the hypothesis that only a single kinesin molecule is required for movement. It can be shown that, if moving a microtubule required several kinesin molecules situated along its length, then the probability of a microtubule moving a distance greater than its length would increase abruptly from zero to unity as the kinesin density was raised above the critical value. The probability instead increased gradually, as expected if one kinesin molecule suffices (Fig. 3*b*). At kinesin densities less than $\sim 20 \mu\text{m}^{-2}$, each microtubule had a low probability of moving more than its length, presumably because there was only a slight chance that it encountered another kinesin molecule before detaching; at these densities we believe that movement was due to single kinesin molecules. From the data of Fig. 3*b*, we estimate that at a density of $60 \mu\text{m}^{-2}$, the average distance between kinesin molecules along the path of a microtubule is equal to the mean length of the microtubules ($2.5 \pm 1.4 \mu\text{m}$, mean $\pm$ s.d., $n=233$). If only half of the kinesin molecules are correctly oriented with respect to the microtubule's axis, then the distance over which a kinesin molecule can reach and react with a microtubule may be estimated at

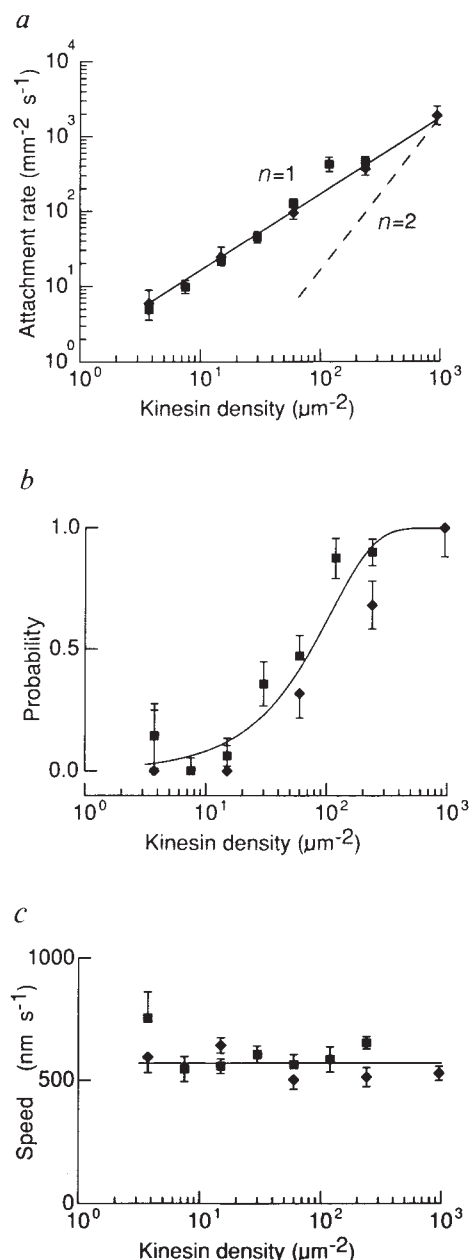


FIG. 3 *a*, Dependence of the microtubule attachment rate upon the kinesin density on a glass surface. The solid line, with a slope of one in this doubly logarithmic plot, is the relation expected if one kinesin molecule sufficed to move a microtubule; for comparison, the dashed line, with a slope of two, represents the result expected if attachment and movement required the cooperation of two kinesin molecules. *b*, The relation between the probability that a microtubule moves a distance greater than its length and the logarithm of kinesin density. The solid line is a plot of the equation $1 - \rho e^{-\rho} / (1 - e^{-\rho})$, in which ρ is the kinesin density divided by $60 \mu\text{m}^{-2}$; this equation corresponds to the prediction of a model in which a single kinesin molecule suffices to move a microtubule (J.H., in preparation). *c*, Dependence of the speed of microtubule movement upon kinesin density. Speeds of microtubule motion were measured from drawings by hand on acetate-sheet overlays of taped video images acquired at 33 ms intervals. At low kinesin density, the speed was determined as the rate at which the microtubule's trailing end approached the fixed (nodal) point at which the kinesin molecule was located. The glass surface was pretreated with tubulin and cytochrome *c* as described in the legend to Fig. 1. The data in *a*, *b* and *c* correspond to observations from a total of 233 individual microtubules in two different experiments (distinguished by symbols). The ATP concentration was 1 mM. Before being infused into the chamber, the diluted microtubules were thrice passed through a 30-gauge needle to shear them into filaments of length $2.2 \pm 1.4 \mu\text{m}$ (mean $\pm$ standard deviation, $n=233$). The error bars correspond to standard errors of the means.

13 nm. This distance, which is compatible with the dimensions of kinesin molecules and microtubules, is a lower limit because the calculation assumes that all the kinesin molecules on the surface are functional.

The greatest microtubule-moving activity in the low-density assay co-fractionated with the 9S peak of kinesin^{8,9} on a sucrose-density gradient containing 4 μM cytochrome *c* (data not shown). Individual kinesin molecules can therefore elicit movement. This finding in conjunction with the several lines of evidence described above—movement at low kinesin density, attachment of a microtubule at one point on the substrate and the attachment and detachment rates—indicates that a single kinesin molecule is sufficient to move a microtubule. One possible reservation is that kinesin might preferentially aggregate at the glass surface; the binding of one kinesin to the surface might then nucleate the binding of others. But the formation of such complexes is an unlikely prerequisite for movement because, at low kinesin concentrations, aggregation would necessarily be slow and incomplete. For example, if kinesin's hypothetical association occurred with a rate constant of $10^7 \text{ M}^{-1}\text{s}^{-1}$, a value approaching the diffusion limit and greater than the on-rate for tubulin polymerization¹⁹, then at the lowest kinesin concentration used in Fig. 3 (100 μM), less than 5% of the bound kinesin would occur in aggregates. Because no deviation from linearity is seen in Fig. 3, we conclude that the functional motor consists of a single kinesin tetramer.

Determinants of microtubule movement

Measurements of microtubule motion at high and low kinesin densities show how the speed of movement is influenced by the number of motor molecules interacting with the microtubule. At an almost saturating ATP concentration (1 mM), the speed of microtubule movement was independent of the kinesin density (Fig. 3c); we infer that one kinesin molecule can move a microtubule as quickly as can several molecules. At kinesin densities $<20 \mu\text{m}^{-2}$, at which microtubules are being moved by single kinesin molecules, the speed was independent of the microtubule length, which ranged from 0.7 to 7 μm .

As the ATP concentration was reduced, the speed of movement declined and became dependent on the number of participating motors. For low kinesin densities, at which we expect only one kinesin molecule to be involved in a microtubule's movement, the speed followed the Michaelis-Menten equation²⁰; at low ATP concentrations, the Hill coefficient was one (Fig. 4). This result is anticipated if just one kinesin molecule hydrolyses a single ATP to produce an incremental microtubule movement. On the other hand, when the kinesin density was raised to levels at which we expected several molecules to contribute to a microtubule's movement, we obtained a seemingly paradoxical result: the more kinesin molecules that participated, the slower was the motion (Fig. 4). Although at high ATP concentrations the maximal speed was independent of kinesin density, at lower ATP concentrations the speed depended on kinesin density, so that several motors moved a microtubule more slowly than did a single motor. This effect became more pronounced as the ATP concentration was reduced; the Hill coefficient exceeded unity. Control experiments showed that the decrease in speed with decreasing ATP concentration was not caused by depletion of ATP by kinesin's hydrolytic activity (see Fig. 4 legend). Instead, the simplest explanation for this behaviour is that kinesin heads in rigor, those bound to the microtubule but lacking ATP, impeded the movement produced by active heads interacting with the same microtubule. Such an explanation has been considered to account for the noncompetitive inhibition by adenylyl-5'-yl imidodiphosphate (AMPPNP) and vanadate of the speed at which sea-urchin-egg kinesin moves microtubules¹⁵. Because movement persisted at low ATP concentrations, however, this impediment must have been incomplete, perhaps because the bound kinesin molecules were quite

compliant or because the rigor bonds could be broken by active heads.

Implications for the reaction cycle

The speed of microtubule motion produced by a single kinesin molecule places limits on the force and displacement generated during each cycle of ATP hydrolysis. Because one motor molecule can move a microtubule as quickly as can several, the force exerted by a single kinesin molecule must significantly exceed the viscous drag from the surrounding fluid. The drag force acting on a microtubule moving across the surface at a speed of 600 nm s^{-1} is about 2–3 fN per micrometre of microtubule length²¹. A single kinesin molecule must produce a force in excess of 60 fN to account for the measured lack of dependence of speed on microtubule length. The force thought to be produced by a molecule of muscle myosin^{1,7} (500 fN) is thus of the same order of magnitude as what we consider a reasonable lower limit for the force produced by a kinesin molecule.

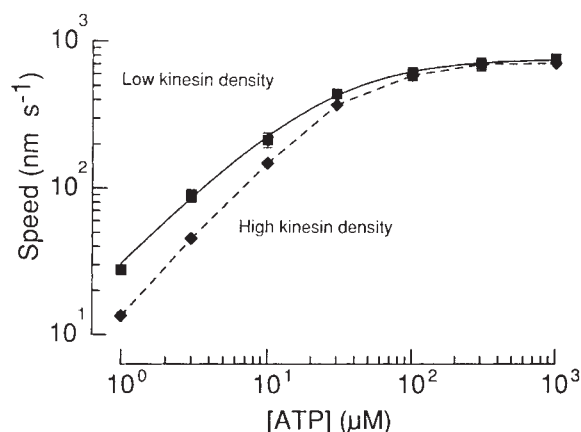


FIG. 4 Dependence of the speed of microtubule movement upon ATP concentration. The kinesin densities were $24 \mu\text{m}^{-2}$ (■) and $\sim 2400 \mu\text{m}^{-2}$ (◆). METHODS. The experimental protocol was identical to that of Fig. 1b except that the nucleotide concentration of the microtubule-containing buffer solution was reduced to $<1 \mu\text{M}$ by a double application of the following procedure: microtubules were diluted 100-fold into nucleotide-free buffer solution, centrifuged for 300 s at 10^6 m s^{-2} , and the pellet was then resuspended in nucleotide-free buffer. In control experiments in this nominally nucleotide-free solution, microtubules bound to the kinesin-coated surface but movement was undetectable at a resolution limit of 0.5 nm s^{-1} , or one-twentieth of the speed measured in the presence of $1 \mu\text{M}$ ATP. ATP was added to the nucleotide-free buffer at the stated concentrations. All speed measurements were made from observations of 6–21 individual microtubules; standard errors of the means are indicated by error bars except when smaller than the symbols. To rule out the possibility that the lower speeds measured at high kinesin density and low ATP concentration were caused by the hydrolysis of ATP by kinesin, the speed was measured over a period of 300 s beginning about 30 s after adding the microtubules to the chamber. Only at the lowest ATP concentration ($1 \mu\text{M}$) was there a significant slowing of the microtubules over time: the fractional decrease in speed over time was $0.0011 \pm 0.0004 \text{ s}^{-1}$ (mean $\pm$ standard error, $n=10$) and led to an error of less than 10%. Maintenance of an ATP concentration of $1 \mu\text{M}$ with an ATP-regeneration system (2 μM creatine phosphokinase and 2 mM phosphocreatine) had no effect on the measured speed. At low kinesin density the probability that a microtubule moved a distance greater than its own length was 0.22 ± 0.06 (mean $\pm$ standard error, $n=50$), confirming that movement was mainly due to single kinesin molecules (see Fig. 3b). At high kinesin density it is estimated that 10–40 kinesin molecules were moving each microtubule. From Fig. 3b, a density of $\sim 60 \mu\text{m}^{-2}$ corresponded to an average of one kinesin molecule per microtubule; we accordingly expect about 40 molecules per microtubule at a density of $2400 \mu\text{m}^{-2}$. By the model discussed in the legend to Fig. 3, a lower limit of about 10 kinesin molecules per microtubule follows from the observation that even the shorter microtubules ($<2 \mu\text{m}$ long) had only a small probability of dissociating (<0.1) before they had moved ten times their own length.

It is remarkable that the speed of microtubule movement at high ATP concentrations does not depend on the number of participating kinesin molecules (Fig. 3c). Consistent with this observation, the speed at high kinesin density and over a wide range of ATP concentrations is essentially independent of microtubule length, and hence of the number of kinesin molecules involved. Because microtubule movement can be achieved by a single kinesin molecule, knowledge of the ATPase activity of kinesin in our assay would allow determination of the step size, or the displacement per cycle of ATP hydrolysis. If the microtubule-activated ATPase were identical to that in solution in the presence of high ATP and microtubule concentrations^{22,23}, $V_{\max} = 20\text{--}30\text{ s}^{-1}$, then a maximal speed of $500\text{--}750\text{ nm s}^{-1}$ would imply a step size of $15\text{--}30\text{ nm}$. This tentative value constitutes a big conformational change that exceeds the 4-nm estimate made from video recordings⁴.

An important corollary of our conclusion that one kinesin molecule can move a microtubule is that a single molecule can also confine a microtubule at the glass surface. During movement at low kinesin density and high ATP concentration, the microtubule is strongly associated with the surface; in the experiment of Fig. 3, for example, there was a 70% probability that a microtubule's trailing end reached the nodal point at which the motor was located. It may be calculated from our results that one kinesin molecule can maintain its grip on a microtubule over $5 \pm 2\text{ }\mu\text{m}$, which is equivalent to a period of $9 \pm 3\text{ s}$ or to some 200–1,000 cycles of ATP hydrolysis. It is possible that kinesin's two globular heads work hand-over-hand, so that one

is always bound and prevents the microtubule from diffusing away. Alternatively, the two heads may work independently, as is suggested by experiments using single-headed myosin^{7,24,25} and dynein^{17,26}. If this is so, the time in the reaction cycle during which the kinesin heads are detached from the microtubule must be so brief, probably less than 1 ms , that the microtubule is unlikely to diffuse out of reach of the kinesin molecule. In either case, the ability of one kinesin molecule to hold and move a microtubule may represent a functional adaptation; during organelle transport, in which it is thought that only a few kinesin molecules produce movement²⁷, detachment would be disadvantageous because diffusion of the organelle away from a microtubule would greatly reduce the speed of transport.

Conclusion

In this study, we describe an assay for measurement of the motile properties of individual motor molecules. Experimental systems of this sort provide information that studies of populations of molecules cannot; it should be possible, for example, to detect heterogeneity in populations of derivatized or enzymatically cleaved motor molecules. In conjunction with high-resolution systems for measuring force and displacement, such assays should also allow analysis of the force-generating step, an event that has been difficult to resolve using larger populations of motor proteins. Like patch-clamp recording from ion channels²⁸, the study of movement produced by single motor molecules provides an assay sensitive enough to monitor the activity of an individual protein molecule. □

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LETTERS TO NATURE

A millisecond pulsar in a 32-minute binary orbit

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WE report the discovery of a millisecond pulsar in the globular cluster 47 Tucanae (NGC104). It has a spin period of 4.479 ms and is a member of a binary system with an orbital period of 32 min and an eccentricity of 0.32. The mass function, $2.9 \times 10^{-8} M_{\odot}$, is the smallest value for any known binary system. We find that the observations are consistent with a neutron star of mass $1.4 M_{\odot}$ and a white-dwarf companion of mass $0.8 M_{\odot}$. The millisecond spin period combined with the very short orbital period offers, for the first time, the possibility of observing spin-orbit coupling effects predicted by general relativity theory.

We began a search for millisecond pulsars in globular clusters using the Parkes radio telescope in November 1987. We observed at frequencies of 430 and 610 MHz using dual-polarization receivers with system-noise equivalents $\approx 120\text{ Jy}$. The receiver pass bands were resolved into $32 \times 33\text{ kHz}$ or $24 \times 250\text{ kHz}$ channels per polarization. In each case signals from the two polarizations were summed after detection, filtered and sampled every $250\text{ }\mu\text{s}$ with only the signal polarity being recorded (one-bit quantization). At each telescope position we took samples continuously for 10^4 s and recorded them as a single data set. We combined the outputs from the filter channels after correcting for the relative time delays that are due to interstellar dispersion. We repeated this for a range of dispersion measures and searched for periodicities at each dispersion using power spectrum analysis followed by superposed epoch analysis at selected periods. The search was sensitive to periods in the range $0.5\text{--}40\text{ ms}$ for dispersion measures up to $200\text{ cm}^{-3}\text{ pc}$.

We discovered¹ two millisecond pulsars in the direction of 47 Tuc (right ascension (1950) = $00^{\text{h}}21^{\text{m}}54^{\text{s}}$, declination (1950) = $-72^{\circ}21'00''$) the first cluster observed. Here we give details of the short-period binary pulsar PSR0021-72A; details of the other

pulsar will be reported elsewhere. We first saw the pulsar signal as a peak near 223 Hz in the power spectrum, but folding the data at this frequency did not yield a significant pulse. However, when it was folded in short subsets and plotted (Fig. 1a) we recognized a pattern characteristic of a pulsar in a binary orbit. The orbital period is only 32.4 min, an order of magnitude shorter than that of any other known binary radio pulsar. When we used a suitable orbital model to remove the drift in the pulse phase from subset to subset (Fig. 1b) the data could be summed to give the pulse profile for the full record (Fig. 1c). The observed parameters of the pulsar are given in Table 1. Because of the short binary period and weakness of the pulsar, the analysis techniques used for other binary pulsars, such as those in ref. 2, are inappropriate. We must integrate the data over several orbital periods to obtain a single profile in the manner illustrated by Fig. 1.

Although the beam diameter of the radio telescope (≈ 25 arcmin at 610 MHz) is not small enough to locate the

pulsar within the cluster, we are confident of the association. The dispersion measure agrees with the expected value for the cluster distance (5 kpc) and direction. The galactic latitude of the cluster is large (-45°) and the region is uncluttered. The second pulsar discovered in the same radio beam¹ has, within the measurement uncertainty, the same dispersion measure. Two weak, unresolved radio sources with fluxes similar to the pulsars have been detected within the central region of the cluster in synthesis maps made at the Molonglo radio telescope (A. J. Turtle, personal communication).

We observed the pulsar over four observing periods (20–23 November 1987, 22–29 February 1988, 5–7 July 1988, 9–16 August 1988) and recorded a total of 27 data sets. For analysis of each record we adopted a keplerian model defined by the parameter set $\mathbf{K} = (x, e, T_0, P_b, \omega)_{\text{Kep}}$. Here $x = (a_1/c) \sin i$, a_1 is the semi-major axis of the pulsar orbit, i the orbital inclination, e the eccentricity, T_0 the epoch of periastron, P_b the binary period and ω the longitude of periastron. The model gives the light travel time across the orbit (the Römer delay) $\Delta_R(\mathbf{K}, t)$ shown as the curved lines in Fig. 1a.

The pulsar signal strength varies widely from one observation to another and even within a single 10^4 -s observation, but even in the best data sets the pulsar peak after folding is never greater than 10σ of the baseline noise; about one third of the data sets show no statistically significant signal. The intensity variation is not inconsistent with interstellar scintillation, but intrinsic variability cannot be ruled out.

This pulsar has been detected at both 430 MHz and 610 MHz, the observed dispersion measure being the same at both frequencies to within the measurement accuracy of about $\pm 2 \text{ cm}^{-3} \text{ pc}$. No periodic signal near 223 Hz has ever been seen at sky positions other than that of 47 Tuc during our globular cluster survey, though we readily detected other known millisecond pulsars (such as those in M4 and M15). We could determine the pulsar period to an accuracy of ~ 200 ps (1σ) in individual data sets and observed the expected Doppler variation that is due to the motion of the Earth relative to 47 Tuc to the same accuracy, even though the Doppler variation over the nine-month span of the observations is more than 10^3 times larger.

To fit the data we used the model to reduce, by folding, the entire data set to a single pulse profile which we then convolved with an idealized pulse-profile template. We took the peak height after convolution (which is proportional to the peak flux in the

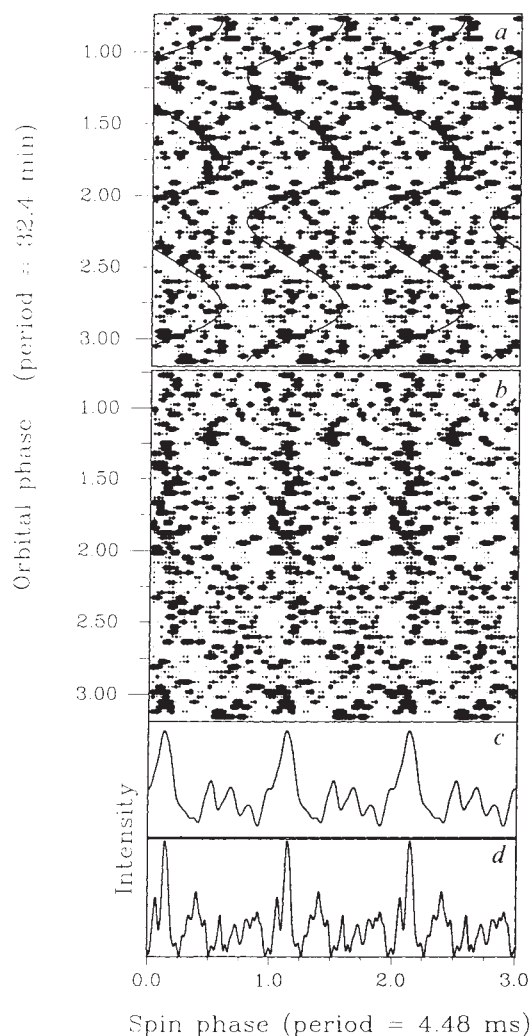


FIG. 1 Subsets of data folded on the mean pulse period, each being displayed three times for clarity. Each subset comprises ~ 1 min (15,000 pulse periods) of data. Signal strength is represented by dot size. *a*, Curved lines show the variation of the Römer delay derived from the keplerian model fitted to the data. *b*, Each subset has been shifted by Δ_R , bringing the pulse into a vertical column. *c*, Sum of all subsets, the relative-intensity profile of the integrated pulse. Signal strength varies widely from record to record and even within individual records. Data displayed here is from two of the best records. Data in *a*–*c* recorded on 22 November 1987 (the record in which the pulsar was discovered). *d*, Integrated profile recorded on 22 February 1988 (at different frequency and twice the sampling rate).

TABLE 1 Parameters of the binary pulsar PSR0021-72A

Observed parameters		
Spin period	P	$4.4789545 \pm 1 \text{ ms}$
Spin period derivative	$\dot{P}$	$< 10^{-15} \text{ s s}^{-1}$
Dispersion measure	DM	$67 \pm 2 \text{ cm}^{-3} \text{ pc}$
Mean flux density at 430 MHz	S_{430}	$4 \pm 2 \text{ mJy}$
Fitted parameters		
Projected semi-major axis/ c	x	$2.38 \pm 3 \text{ ms}$
Binary period	P_b	$1,942.0845 \pm 20 \text{ s}$
Eccentricity	e	0.32 ± 1
Longitude of periastron	ω_0	$248 \pm 5^\circ$
Epoch of periastron	T_0	$2,447,214.8606 \pm 2 \text{ JD}$
Apsidal rate	$\dot{\omega}$	$0.565 \pm 20^\circ \text{ d}^{-1}$
Amplitude of Einstein delay	γ	$510 \pm 30 \text{ } \mu\text{s}$
Derived parameters		
Pulsar mass	M_1	$1.38 \pm 8 \text{ } M_\odot$
Companion mass	M_2	$0.77 \pm 5 \text{ } M_\odot$
Mean separation	a	$300,900 \pm 4,000 \text{ km}$
Orbital inclination	i	$0.38 \pm 1^\circ$

The uncertainties indicated in the final digits are estimated 90% confidence limits.

pulsar signal) as the figure of merit to be maximized by varying the model parameters. Because of the relatively low signal-to-noise ratio and the large number of free parameters in the model, we took care not to optimize the model on a false, non-global, peak figure of merit in the parameter space. We optimized the parameters in two stages, the first being a very extensive grid search over the multi-dimensional parameter space. We searched all angle-like parameters over a full rotation, the eccentricity over the range $0 \leq e < 1$ and other parameters over ranges as much as twice their anticipated values. Once we located the global maximum, we used standard multi-dimensional optimization methods for the final refinement of the parameters. Figure 2 shows the results for some of the more important parameters for two data sets.

Two effects unobservable in individual records should be evident over the longer times between observing periods, an increase in ω (the apsidal advance) that is the result of both classical and general relativistic effects³ and a periodic variation in pulse arrival time, Δ_E , the Einstein delay. Δ_E is the sum of two contributions, the change in gravitational redshift and the change in the transverse Doppler shift of the pulsar period that is due to the periodic variation in orbital separation and velocity

when $e \neq 0$. $\Delta_E(u) = \gamma \sin u$ where u is the eccentric anomaly. For single observations Δ_E is inseparable from Δ_R . Because Δ_R depends on ω and Δ_E does not, however, the advance of ω allows a separation of the two effects and a determination of γ (the amplitude of the Einstein delay).

To account for the relativistic parameters $\dot{\omega}$ and γ , we fitted a relativistic model⁴ with parameters $\mathbf{R} = (x, e, T_0, P_b, \omega, \dot{\omega}, \gamma)_{\text{rel}}$ to the keplerian parameters derived from individual records. We have determined the parameters with sufficient accuracy to track the orbital phase without ambiguity over the nine-month period spanned by the data. However, we could not connect the spin phase from one record to the next and so the pulsar period that we used was the mean of determinations from each record. The effect of $\gamma \neq 0$ is to cause $a_1 \sin i = xc$ to seem to vary with the apsidal period. Figure 2b shows the observed variations in ω and $a_1 \sin i$ between two representative data sets taken in November 1987 and February 1988. The implied trends in ω and $a_1 \sin i$ are confirmed by August 1988 data. The fitted relativistic parameters from all data sets are listed in Table 1.

Measurements of $\dot{\omega}$ and γ allow us to determine the masses of the pulsar and its companion, M_1 and M_2 . Figure 3 shows lines of constant $\dot{\omega}$ and γ in the $M_1 M_2$ -plane for the extreme values of these parameters allowed by the fit of the relativistic model to the binary orbit. We calculated the classical contributions to $\dot{\omega}$ from a white-dwarf model^{3,5} with $\mu_e = 2$ (the baryon-to-electron ratio). These classical effects dominate the $\dot{\omega}$ curves for $M_2 < 0.5 M_\odot$. The range of possible pulsar masses includes the Chandrasekhar mass, $1.4 M_\odot$. Other system parameters derived from this result appear in Table 1. The deduced pulsar mass is in agreement with the only other precise pulsar mass determination of $1.42 M_\odot$ and $1.40 M_\odot$ for the stars in the PSR1913+16 binary system⁶. The derived companion mass of $\sim 0.8 M_\odot$ corresponds to a radius $\approx 7 \times 10^3$ km, much smaller than the radius of its critical Roche lobe⁷ which has a mean radius $\approx 105 \times 10^3$ km. We calculate the classical component of $\dot{\omega}$ to be $\sim 10^{-3} \text{ deg d}^{-1}$, about 0.2% of the relativistic component.

The angle of inclination of the orbit to the plane of the sky is surprisingly small: the orbit is viewed almost exactly face-on. Had the inclination been much greater, however, the large variation in the arrival time because of the Römer delay would have made it difficult to fold the pulsar signal, notwithstanding a significant peak in the power spectrum. Because the 47 Tuc power spectrum does not show any other unidentified significant peaks, we think it unlikely that there are a large number of undetected short-period binary pulsars in the cluster. It is reasonable to assume that the small orbital inclination is simply fortuitous.

The observed pulse duty cycle of ~ 0.05 , typical of most pulsars, suggests that the pulsar beam makes a fairly large angle with the spin axis⁸. If so, given the very small orbital inclination, then the spin axis is not aligned with the orbit normal and we might be able to see precession of the pulsar arising from relativistic spin-orbit coupling^{9,10} (which here dominates the classical quadrupole coupling). We calculate the precession period to be ~ 8 yr based on 10^{45} g cm^2 for the pulsar moment. Such precession could also make observable the aberration delay, giving the first direct evidence of pulsars as rotating beacons⁴.

Synchronization, alignment and circularization timescales¹¹ are much greater than the cluster age ($\sim 10^{10}$ yr). Ablation of the companion by pulsar radiation¹² should be negligible. However, the secular change in the orbit that is due to gravitational radiation¹³ implies $e \geq 0.8$ as recently as 5×10^7 yr BP (before present) and coalescence of the stars in only $\sim 10^6$ yr, so the system lifetime is very short compared with the cluster age. This short lifetime, the present highly elliptical orbit and the probable misalignment of the spin and orbit present difficulties in accounting for the system's formation.

According to the standard theory for the formation of millisecond pulsars¹⁴, an old neutron star accretes matter from a

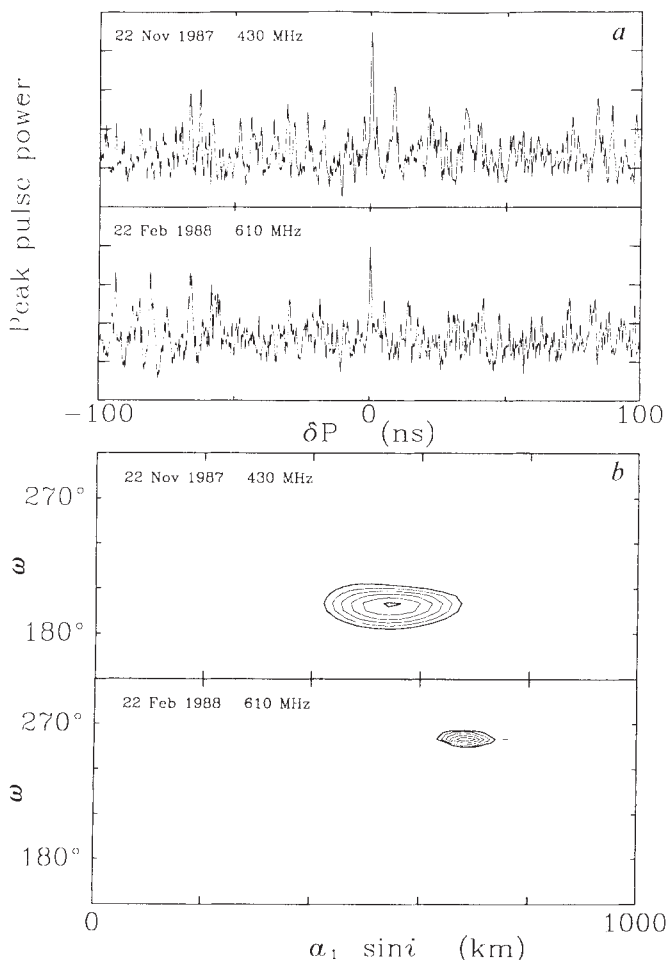


FIG. 2 a, Peak pulse power (figure of merit) plotted against deviation of the spin period from the nominal barycentric period for two 47 Tuc data sets. The period origin in the lower panel has been shifted by 124.7 ns, the calculated offset that is the result of the Earth's orbital and diurnal motion. b, Contours of the figure of merit for the same data sets on a two-dimensional plot through the six-dimensional space of the model parameters. In both cases the contour interval is 1σ and contours above the 4σ significance level are shown. The change in both ω and $a_1 \sin i = xc$ between November 1987 and February 1988 is evident. We note that the higher sampling rate used in the February 1988 observation allows more precise determination of the parameters.

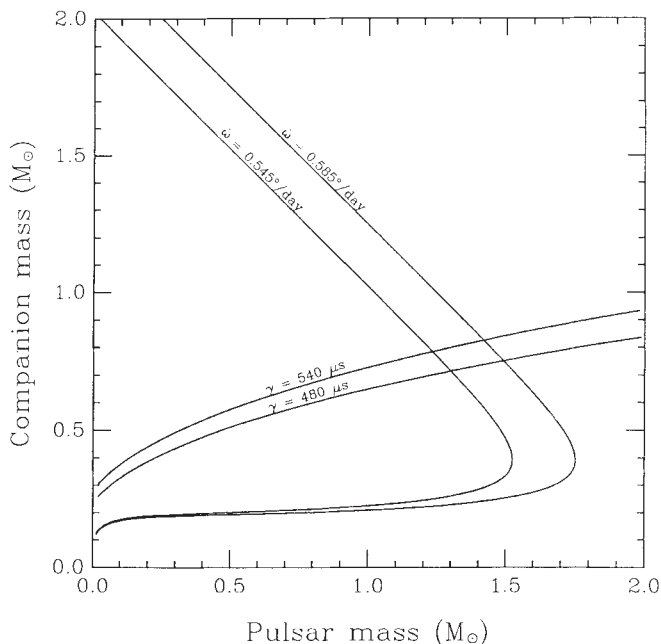


FIG. 3 Lines of constant $\dot{\omega}$ and γ for the 90% confidence bounds of these parameters and the fitted values of e and P_0 (Table 1). These curves show how the stellar masses are constrained by the parameters of the relativistic model. The allowed masses lie in the quadrilateral centred on the point $M_1 = 1.38 M_\odot$, $M_2 = 0.77 M_\odot$ which is formed by the intersection of the lines. The nearly horizontal sections of the $\dot{\omega}$ lines arise from the tidal interaction between the pulsar and a low-mass white dwarf.

binary companion, increasing its rotation rate to form a 'reborn' pulsar. This transfer of orbital angular momentum to the pulsar leads to the spin being aligned with the orbit since the initial angular momentum of the pulsar is negligible compared with its final value. The orbit rapidly becomes circular because of the strong tidal interaction associated with the mass transfer¹¹.

An alternate formation theory¹⁵ involves mass transfer from a binary companion onto a white dwarf, increasing its rotation rate and ultimately causing it to exceed the Chandrasekhar mass limit and collapse to a rapidly spinning neutron star. Although the orbit may become eccentric if the collapsing star sheds mass, $e \geq 0.32$ requires $\Delta M \geq 0.7 M_\odot$ so that before collapse $M \geq 2.1 M_\odot$, too large for a white dwarf. Again, spin and orbit become aligned.

Neither formation theory is consistent with the present state of this system. It could, however, have resulted from a relatively recent ($\sim 10^7$ yr BP) strong encounter of a reborn pulsar, in circular orbit with its mass-donating companion, and a third star. This is not unlikely; estimates¹⁶ of the mean waiting time for such an event are of the same order as the cluster age. The negative binding energy of the binary is $\sim 10^3$ times greater than the average kinetic energy of the cluster members, so an interaction producing $e \geq 0.32$ is very likely to have been a resonance scattering¹⁷ in which a three-body system is formed that lasts for a number of chaotic orbits before ejecting one member, probably the lightest. The probable outcome would have been to decrease a , greatly increase e and randomize the spin orientations relative to the final orbital plane. $\square$

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Molecular outflows in protostellar evolution

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MOLECULAR outflow is an energetic mass-ejection phenomenon associated with very early stage of stellar evolution^{1,2}. The large kinetic energy involved in the phenomenon indicates that outflow may play an essential role in the process of star formation, particularly by extracting angular momentum³. Most of the previous searches have been strongly biased toward optical or near-infrared signposts of star formation¹. They are not able, therefore, to provide the complete database necessary for a statistical study of the evolutionary status of molecular outflow. To overcome this difficulty, it is of vital importance to make an unbiased search of single molecular clouds for molecular outflows; here we report the final result of such a survey of the Lynds 1641 dark cloud. We show that molecular outflows are characterized by a total luminosity significantly greater than that of T Tauri stars. This indicates that molecular outflow corresponds to the main accretion phase of protostellar evolution, in which the luminosity excess is due to the gravitational energy released by dynamical mass accretion onto the protostellar core⁴.

L1641 is the nearest (480 pc) giant molecular cloud in Orion showing evidence of extensive recent star formation^{5–7}, and therefore providing a unique opportunity to carry out the statistical study required to address the evolutionary status of molecular outflow. We focus on the area of L1641 at declination $(1950) \leq -7^\circ$, excluding the Orion nebula, to avoid confusion in the infrared wavelength. To make a complete survey of outflows above a certain mechanical luminosity limit in the cloud, we used three radio telescopes, the 4-m (Nagoya University), 4.9-m (University of Texas) and 45-m (Nobeyama) telescopes, in the period 1985–1989 (refs 8, 9 and Y. Fukui *et al.*, manuscript in preparation). They have beam sizes of $2.7'$ (at 115 GHz), $1.3'$ (at 230 GHz), and $17''$ (at 115 GHz), respectively. Observations were initiated by a large-scale unbiased survey of the L1641 cloud⁸ with the 4-m telescope in CO and ^{13}CO ($J=1-0$) emission, and were followed by detailed mapping of the regions exhibiting high-velocity CO emission with the three telescopes¹⁰. The survey has discovered and mapped five molecular outflows^{8,9} as listed in Table 1 along with one outflow reported in the literature¹¹. They are located in a ^{13}CO integrated-intensity map in Fig. 1. All the outflows have been found to be associated with far-infrared point sources detected by the Infrared Astronomical Satellite (IRAS)¹². The detection limit of the present study corresponds to $\sim 1 \times 10^{-3} L_\odot$ in mechanical luminosity, two orders of magnitude smaller than that of L1551-IR55, a typical molecular outflow in a dark cloud¹³. We note that there could be undetected outflows having mechanical

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TABLE 1 Molecular outflows in the L1641 dark cloud

Outflow	Size* (pc)	Velocity† (km s ⁻¹)	Age‡ (10 ⁵ yr)	Mechanical luminosity§ (L _☉)	Bolometric luminosity (L _☉)	IRAS source	Reference
1. L1641-Centre	0.8	7	1.1	0.006	40.2	05363-0702	9
2. Haro4-255	0.7	13¶	0.5	0.12	60.5	05369-0728	11
3. L1641-South	1.0	14	0.7	0.013	294.7	05380-0728	8
4. L1641-South3	0.6	23	0.25	0.017	123.1	05375-0731	present
5. L1641-South4	0.8	8	1.0	0.003	20.2	05384-0808	present
6. L1641-South2	0.6	25	0.25	0.037	20.6	05403-0818	9

* Maximum radius of the outflow lobe from the IRAS point source.

† Maximum relative velocity from the quiescent cloud.

‡ Dynamical timescale determined from the size and the velocity.

§ Geometrical average of the upper and lower limits (refs 24, 25).

|| Ref. 14.

¶ The averaged relative velocity of blue and red wings calculated from the literature.

luminosities less than the above limit: (1) outflows having smaller velocity extents may be masked by the local line width of the ambient CO cloud; (2) weak outflows below the present sensitivity cannot be detected; and (3) compact outflows in the earliest phase of evolution may be missed because of beam dilution.

We have used the present sample of outflows to elucidate the characteristics of the IRAS sources that are driving outflows. Forty-eight IRAS sources detected at 12, 25 and 60 μ m are selected from a list of the IRAS point sources in L1641 (ref. 14). A two-colour diagram of the 48 sources (Fig. 2a) shows that all the outflows are located in the lower left part of the diagram, indicating that the outflows are characterized by significantly lower dust-colour temperature than T Tauri stars characterized by a rectangular box in Fig. 2a (refs 15, 16). Most of the IRAS sources without outflow are located in or near this box. Here we shall tentatively suppose the IRAS sources without outflow as T Tauri stars, although some of them may be found as outflows in a future more sensitive survey. A close inspection of the IRAS flux data indicates that 48 sources have nearly uniform 12- μ m fluxes, and that 25- μ m fluxes of the outflow sources are considerably greater than those of the T Tauri stars (Table 2). The excess in the 25- μ m flux is therefore the origin of the cold infrared spectrum of the outflow sources. The 60- μ m flux shows basically the same trend as the 25- μ m flux. The difference in 12/25- μ m colour temperature indicates that the outflow sources have dust envelopes that are ~ 10 times more massive than the T Tauri stars according to a two-temperature fitting of the IRAS spectra⁹. The outflow sources are thus embed-

ded more heavily in dust envelopes than the T Tauri stars. The large dust mass of the outflow sources is consistent with recent ¹³CO observations in L1641 indicating that molecular column densities toward IRAS sources with outflow is greater than toward those without outflow (H. Chen, personal communication). This strongly indicates that outflow is a signature of an evolutionary stage earlier than pre-main-sequence stars in the Hayashi track¹⁷ represented by T Tauri stars.

A bolometric luminosity/colour diagram for the IRAS sources is shown in Fig. 2b. The present detection limit is $\sim 1 L_{\odot}$. Most of the T Tauri stars have luminosities in the range 2–20 $L_{\odot}$. On the other hand, all the outflows have considerably larger luminosities of 20–300 $L_{\odot}$. We find that $>80\%$ of the IRAS sources that are brighter than 20 $L_{\odot}$ and colder than 190 K (12/25- μ m dust colour temperature) are driving molecular outflows. In the L1641 region, it is shown that optically studied emission-line stars are mostly T Tauri stars having masses of $\sim 1 M_{\odot}$ (refs 18, 19). Thus, solar-type low-mass stars are the main stellar component in the L1641 cloud. The molecular-outflow sources are distinguished from the majority in their luminosity excess. The outflow sources are thus not only embedded deeply in dense gas but also characterized by a large luminosity excess. This luminosity excess could be explained by the gravitational energy released in the dynamical mass accretion onto the protostellar core. The accretion luminosity may be calculated by using the equation $L_{\text{acc}} = GM_*\dot{M}/R$ where $\dot{M}$ is a mass accretion rate⁴. For a typical mass accretion rate in a giant molecular cloud of $10^{-5} M_{\odot} \text{ yr}^{-1}$, and typical radius for a $1 M_{\odot}$ protostar of $4 R_{\odot}$ (ref. 4), the maximum accretion luminosity is estimated to be $\sim 70 L_{\odot}$. This value is considerably larger than that of T Tauri stars, and explains the large luminosity of the outflow sources.

The timescale ratio of T Tauri stars and outflow sources is estimated to be 7:1 from the number ratio of the 48 sources. The actual number of T Tauri stars is assumed to be even larger than that, by a factor of ~ 2 , as the IRAS detected only dusty T Tauri stars²⁰. We therefore estimate the number ratio to be >10 , including T Tauri stars undetected by the IRAS. This is consistent with a typical dynamical timescale of outflow of $\sim 10^5$ yr (Table 1), less than one-tenth of a T Tauri star age for a $1 M_{\odot}$ star ($2\text{--}3 \times 10^6$ yr) (refs 19, 21). On the other hand, the accretion timescale is 1×10^5 yr if we adopt the above mass-accretion rate. This indicates that for low-mass stars, outflow is taking place over almost the entire main accretion phase that is characterized by large luminosities of $\geq 20 L_{\odot}$. Termination of the outflow is probably due to the dispersal of the placental molecular cloud core²², and this dispersal is made both by the accretion and the outflow. The birthline of T Tauri stars corre-

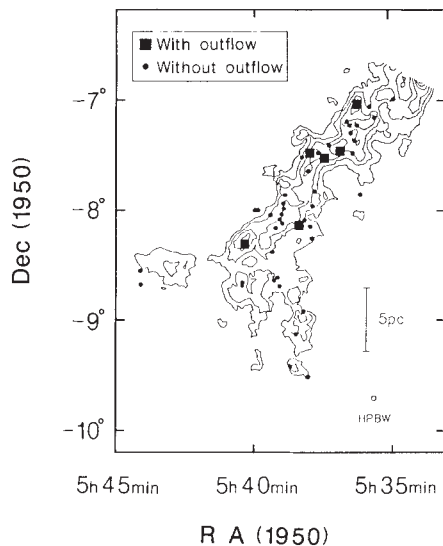


FIG. 1 Contour map of ¹³CO ($J=1-0$) total intensity of the L1641 dark cloud at declination (1950) $\leq -7^\circ$ obtained with the Nagoya 4-m radio telescope. IRAS point sources associated with and without molecular outflow are denoted by filled squares and small circles, respectively. (RA is right ascension.)

TABLE 2 Average flux densities of 48 IRAS sources in L1641

	Number of sources	$\log [F(12 \mu\text{m}) (\text{Jy})]$	$\log [F(25 \mu\text{m}) (\text{Jy})]$
Outflow	6	$-0.19 \pm 0.80 (1\sigma)$	0.91 ± 0.53
T Tauri star	42	-0.26 ± 0.46	0.13 ± 0.49

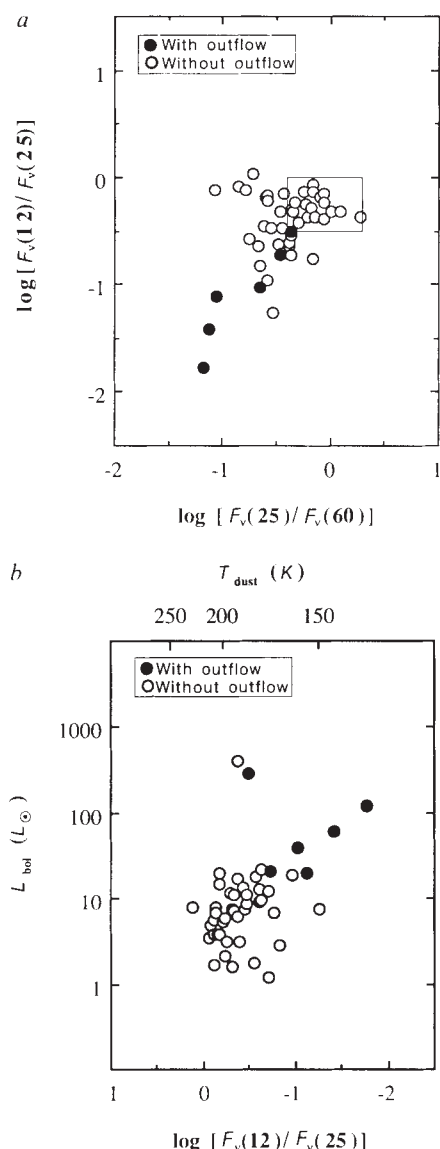


FIG. 2 *a*, A 12/25- μm – 25/60- μm two-colour diagram for 48 IRAS point sources selected in L1641 (ref. 14). A rectangular area corresponds to the T Tauri box¹⁵. Filled circles denote IRAS point sources associated with molecular outflow. *b*, Bolometric luminosity plotted against the 12/25- μm colour diagram for the 48 IRAS point sources. Upper horizontal scale is the dust colour temperature derived from 12- μm and 25- μm flux densities on the assumption of a λ^{-1} emissivity law. Filled circles denote IRAS point sources associated with molecular outflow.

sponds to this epoch, and their ages on the birthline, 1×10^5 yr (ref. 21), are consistent with the timescales of outflow and the main accretion phase.

A consequence of this interpretation is that molecular outflow represents the main accretion phase of a solar-type low-mass protostar. It is then likely that mass accretion takes place inside a flattened circumstellar disk around a protostellar core, with outflow occurring along the poles³. If outflow is ultimately powered by the energy released by the accretion²³, the mechanical luminosity of outflow should be smaller than the bolometric luminosity. In Table 1, we find this to be the case. On the other hand, mass-ejection rate in the outflows in Table 1 is typically $10^{-5} M_{\odot} \text{ yr}^{-1}$, comparable to the mass accretion rate above. This is, however, not a problem in star formation as most of the outflowing mass is actually of interstellar origin ($r \geq 10^{16}$ cm) (ref. 1), and mass flux from the inner circumstellar region ($r \leq 10^{15}$ cm) must be significantly smaller than a mass accretion rate of $10^{-5} M_{\odot} \text{ yr}^{-1}$. □

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Development of Asian monsoon revealed by marked ecological shift during the latest Miocene in northern Pakistan

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CARBON isotopes from soil carbonate^{1–4} and soil organic matter^{5,6} yield palaeoecological information because the carbon in the soil carbonate forms in isotopic equilibrium with local soil CO₂ (refs 1, 7), the isotopic composition of which is in turn determined by local plant cover. Siwalik Group sediments in northern Pakistan contain a well exposed palaeosol record spanning the past 18 Myr. Here we report on stable-carbon-isotope results from associated pedogenic carbonate which indicate a dramatic ecological shift from C₃- to C₄-dominated floodplain biomass beginning ~7.4–7.0 Myr ago. The earlier C₃ floodplain biomasses were probably mainly composed of trees and shrubs, whereas C₄ grasslands dominated in the Plio-Pleistocene. Oxygen isotopes also exhibit a shift in the latest Miocene, probably corresponding to a major climate change which may have induced the forest-to-grassland transition. This dramatic ecological shift in the latest Miocene may mark the inception or a marked strengthening of the Asian monsoon system.

Plants display three distinct carbon isotopic groupings. C₃ plants include virtually all trees irrespective of climate, nearly all shrubs and herbs, and grasses favoured by a cool growing season. They average ~–27‰, but display a range from –35 to –20‰ depending on genus, plant longevity, moisture stress, light intensity and other factors^{8,9}. C₄ plants include a few shrubs in the families Euphorbiaceae and Chenopodiaceae, but especially grasses favoured by warm growing seasons. C₄ grasses average ~–13‰. CAM (crassulacean acid metabolism) plants, which include succulents like cactus and some yuccas, are not important components of ecosystems outside deserts, and will not be discussed here. The isotopic compositions of soil organic matter, where preserved, and of soil CO₂ where soil respiration rates are high, are then determined by the proportion of C₃:C₄ plants in the local biomass. Fractionation effects arising from gaseous diffusion and equilibrium exchange produce an enrichment of

$\delta^{13}\text{C}$ ($[(^{13}\text{C}/^{12}\text{C})_{\text{sample}}/(^{13}\text{C}/^{12}\text{C})_{\text{standard}}] - 1$) in soil carbonate of $\sim 15\%$ with respect to soil-respired CO_2 (refs 1, 7, 10). Soil carbonate formed in the presence of a pure C_3 and pure C_4 biomass would then have $\delta^{13}\text{C}$ (PDB) values at 25°C of ~ -12 and $+2\%$, respectively. Oxygen isotopes in soil carbonate have been shown to have a strong positive correlation with the isotopic content of local rainfall¹.

Palaeosols from the well dated¹¹⁻¹⁵ (Fig. 1) Siwalik Group sediments in the Potwar Plateau region are abundant within the fine-grained floodplain facies of large river systems. Palaeosols typically contain pale red to yellow, clay-rich B horizons that have been thoroughly bioturbated and leached of carbonate. Organic A horizons, which often cap Plio-Pleistocene palaeosols, are rare in the Dhok Pathan formation (8.9–5.3 Myr), and are absent in older beds. We took great care in all our sections to sample carbonate nodules associated with this suite of pedologic features, preferably from the soil base (calic horizons). Most, but not all, palaeosols in the section contained pedogenic carbonate.

Our sampling density averaged ~ 1 palaeosol per 130,000 yr over 17 Myr. From the standpoint of both carbon and oxygen isotopes, the record contains three distinct phases.

From 18 to 7.4–7.0 Myr, almost all of the $\delta^{13}\text{C}$ results (using the PDB dating standard) for soil carbonate fall between -13 and -9% (Fig. 2). Organic matter from two A horizons in the Dhok Pathan formation is $\sim 15\%$ more negative than associated carbonate. This is the same isotope difference displayed between the two phases in modern soils¹⁰, indicating that neither com-

ponent has experienced diagenetic alteration. A single soil at the 8.5-Myr level from the Chinji village section yielded results in the range -7 to -5% . The $\delta^{18}\text{O}$ (PDB) of soil carbonate averaged ~ -10 to -9% , but showed a higher standard deviation than carbon (Fig. 3).

From 7.4–7.0 to 5 Myr, the stable carbon and oxygen isotopic compositions of soil carbonate shift towards more positive values (Figs 2 and 3). A carbon shift seems to begin at ~ 7.4 Myr at Kaulial, but values more positive than the -9% maximum typical of older levels do not occur consistently until 6.4 Myr. At Jalalpur the change had commenced by 7.3–7.2 Myr. We are uncertain whether the spread in these dates is real, or the result of small errors in palaeomagnetic age estimates. The shift at Rohtas, not included in Fig. 1 owing to dating uncertainties in the lower portion of the section, begins a few hundred metres below the base of the Gilbert Chron, a position consistent with a latest Miocene age. This shift was completed at Rohtas and at Jalalpur by ~ 5 Myr with the attainment of positive carbon values. The soil carbonate $\delta^{18}\text{O}$ (PDB) also changed by $\sim 3\%$ during this period, attaining values of ~ -7 to -5% (Fig. 3).

From 5 to 0.4 Myr, the carbon isotopes for soil carbonate fall between -2 and $+2\%$. Soil organic matter lies between -14 and -11% (ref. 10). The average isotope separation between organic matter and soil carbonate is $\sim 15\%$, again indicating that no diagenetic alteration of isotopic composition has occurred¹⁰. The soil carbonate $\delta^{18}\text{O}$ (PDB) falls largely between -7 and -5% for this period and for modern soils in the area.

The carbon isotope results from 52 different palaeosols indicate that a pure or nearly pure C_3 biomass dominated floodplains before 7.4–7.0 Myr ago. In modern ecosystems at low latitudes and altitudes, the narrow range of carbon values from this period can only be produced by closed canopy forests with or without an understorey of C_3 shrubs and herbs. C_3 grasses, normally restricted to cooler higher latitude/higher elevation climates, can also be present in the shaded understorey, but their overall contribution to the biomass is small. Where the canopy is broken, however, patches of C_4 -dominated grassland occur owing to the resulting higher irradiance, water stress, and temperatures^{16,17}. C_4 plants were present, as indicated by a single palaeosol from the level of ~ 8.5 Myr, but evidently they were not common. We therefore cannot interpret the floodplain ecosystem as having been a 'mosaic' of mixed grassland and woodland. Our reconstruction is at variance with previous interpretations based on palaeosols that identified a mosaic of habitats, including grasslands, in the lower Dhok Pathan formation at Khaur¹⁸.

The advent of isotopically more positive soil organic matter and carbonate values after 7.4–7.0 Myr marks the gradual

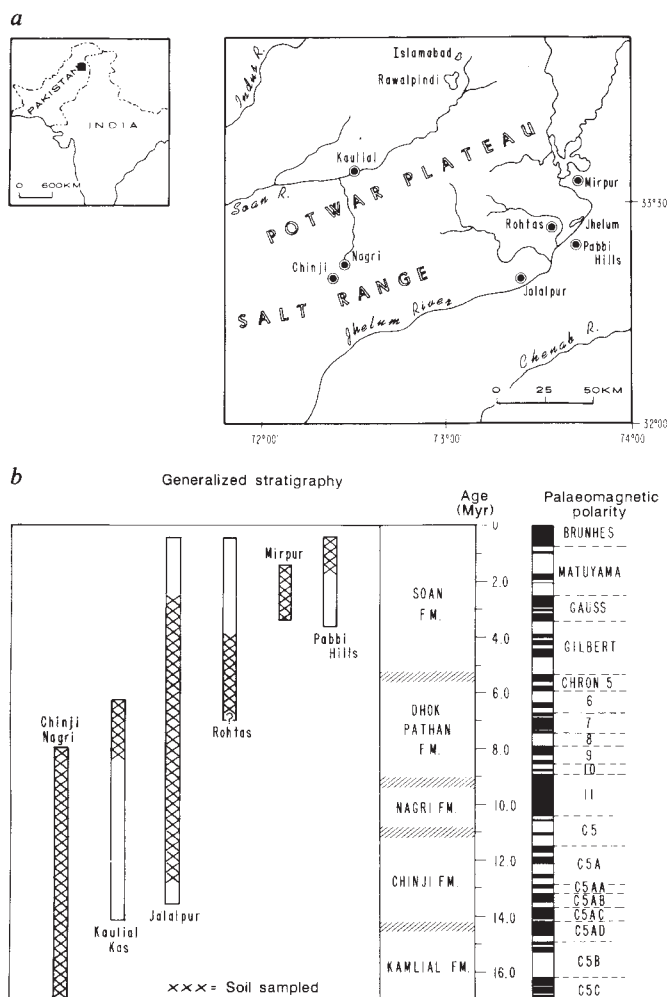


FIG. 1 a, Locations of study sections and b, approximate intervals over which palaeosols were sampled on the Potwar Plateau, northern Pakistan. (Fm. is formation.) Magnetic polarity timescale is from ref. 36.

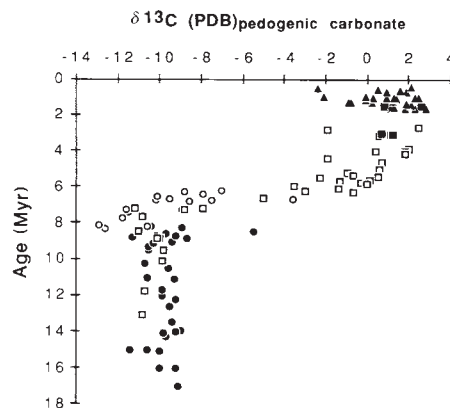


FIG. 2 The $\delta^{13}\text{C}$ (PDB) of palaeosol carbonate nodules plotted against age in the Siwalik Sequence, northern Pakistan. The negative values before 7.4 Myr indicate a C_3 biomass dominated the floodplain, whereas more positive values in the Plio-Pleistocene palaeosols indicate the dominance of C_4 grasslands. ●, Chinji-Nagri; ○, Kaulial Kas; ■, Mirpur; □, Jalalpur; ▲, Pabbi hills.

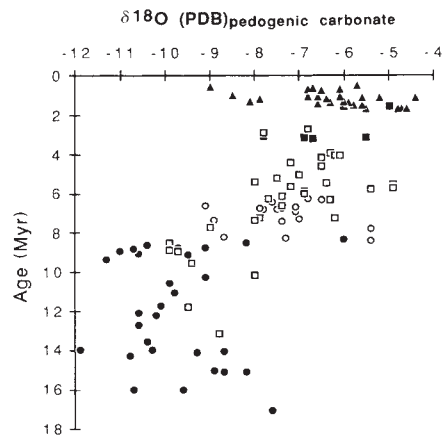


FIG. 3 The $\delta^{18}\text{O}$ (PDB) of palaeosol carbonate nodules in the Siwalik Sequence, northern Pakistan. Oxygen isotopes shift towards more positive values in the late Miocene, indicating a change in the average isotopic composition of soil water probably resulting from regional climatic change. Key, as in Fig. 2.

expansion of C_4 plants on to the floodplain. This must signify the appearance of grasslands because they are the only known C_4 biomasses of large extent. Therefore, a vegetation mosaic of C_4 grassland and C_3 forest was probably present between 7.4–7.0 and 5 Myr. By 5 Myr, C_4 grasses constituted >90% of the biomass, and this persisted through the top of the record (~0.4 Myr). The few negative values obtained perhaps signify limited riparian habitats in which some C_3 trees and shrubs grew. The isotope shift in the carbonates cannot be explained by changes in soil respiration rates over time because soil organic matter also reflects the shift. Moreover, leached zones in Siwalik palaeosols usually exceed 50 cm, consistent with high-respiration-rate soils in which the isotopic composition of carbonate is controlled by the proportion of C_3 : C_4 plants^{1,7,10}.

The reasons for the late Miocene ecological shift can be interpreted in two ways. The shift may have been in response to a climate change that allowed the invasion of C_4 grasses from outside the region. Alternatively, the ecological shift may mark the evolutionary first appearance of C_4 plants, and thus a floral invasion unrelated to a climate change. The oldest C_4 grasses yet documented appear in the late Miocene (~7.0 Myr)¹⁹ of North America, but the palaeobotanical record for grasses is very sparse.

The oxygen isotopes provide important evidence as they shift in every section towards more positive values slightly before the carbon isotope shift. Diagenesis does not seem to have modified the isotopic composition of the carbonates, and the oxygen event is widespread, affecting the entire Potwar Plateau. The isotopic composition of soil water is determined by temperature, the isotopic composition of regional rainfall, seasonality of precipitation, and other factors. Without further evidence we are reluctant to speculate on the exact reason for the shift except to say that a major change in some aspect of the regional climate is indicated.

Moderate rainfall, seasonal drought, and natural fires are all viewed as important to modern grasslands²⁰. One or more of these conditions may have been absent before 7.4 Myr. Higher rainfall in the Miocene, as indicated by thick leached zones in soils, is one explanation. Depth of leaching in modern soils in the area is <50 cm. Leaching depths of 1–2 m (after compaction) in the middle and late Miocene indicate ≥ 1 m of rain per year based on modern patterns²¹. The presence of soil carbonate in palaeosols throughout the record precludes the wet tropical climate called for by Krynine²².

Some soil features, as with isotopic composition, change sharply after ~7 Myr. For example, depth of soil leaching decreases, on average to 50 cm or less. Leached zones (B horizons) are notably yellower (2.5 Y 5/5 dry), in contrast to the strong reds (5 YR 4/4 dry) and oranges of older soils. Most notably, organic A horizons become common in younger sediments. Oxidation during diagenesis may have removed the older humic horizons and altered original soil colours. However, the humic zones and colour changes begin to appear at Kaulial Kas, Jalalpur and Rohtas at ~7.5 Myr, even though burial depths and therefore diagenetic histories differ between sections. Another possible explanation is that the appearance of humic soil zones marks the expansion of grasslands. Of the 17 humus horizons analysed so far, 15 indicate a pure or nearly pure C_4 biomass. In modern soils, organic accumulations under grasslands (mollic epipedons) are considerably greater than under forests²³.

To understand the origins of the vegetation shift in the latest Miocene, it is instructive to look at modern monsoonal climate and its impact on the modern vegetation and soils. Modern climate in northern Pakistan is monsoonal, with 70% of the 40-cm annual rainfall for the Potwar Plateau falling in the warm summer months. This moderate rainfall, the strongly seasonal rainfall distribution, and prevalence of natural fires from summer lightning strikes all favour grasslands over forest²⁰. Mean daily summer growing-season temperatures fall between 30 and

35 °C on the Potwar Plateau, the range favoured by C_4 grasses in particular^{24–27}. Plants listed for western India²⁸ and our own observations on the Jhelum River floodplain indicate that the extant native grasses of the Potwar Plateau region are C_4 s. It is therefore likely that the biomass on floodplains was a largely C_4 grassland before modification by man in the past few millennia.

Our evidence indicates that if the monsoon system already existed it experienced a strong intensification beginning at ~7.4–7.0 Myr, marked by the first appearance of C_4 grasslands favoured by the monsoonal climate of the Potwar Plateau today. The marine record from the northern Indian Ocean supports this, in that diatom assemblages specific to monsoon circulation patterns first appeared 11–10 Myr ago. Abundances of these diatoms increased markedly at 7.3 Myr, an event thought to indicate intensification of the reversing monsoon in the region²⁹.

Our vegetation reconstruction is also supported by mammalian fossil evidence. The larger animals before ~7.0 Myr (tragulids, suids, Okapia-like giraffes, low-crowned bovids and others) were browsers^{30–32}. At 7.4 Myr a major faunal turnover occurred, one which Barry *et al.*³³ speculated was related to climate change. After 7.4 Myr, a grazing adaptation is manifest in the high-crowned dentition of a newly appearing tragulid and large bovids. Overall, the browsers are much less evident in the younger assemblage. Rodents also show a major turnover at ~7.0 Myr, which has been interpreted to be caused by displacement of forests by grassland³⁴. Most significantly *Sivapithecus* sp., which is evidently allied to tree-dwelling orangutans, disappeared from the record at 7.4 Myr³⁵, at about the time grasslands appear. □

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Old carbon in living organisms and young CaCO_3 cements from abyssal brine seeps

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ABYSSAL brine springs at the base of the Florida Escarpment in the Gulf of Mexico ($\sim 3,280$ m, $26^\circ 02' \text{N}$ $84^\circ 55' \text{W}$) are surrounded by communities of abundant heterotrophic organisms^{1–4} and carbonate-cemented crusts⁵. These organisms are apparently supported by local chemosynthetic primary production because the ^{13}C content of their tissues is lower than that found in typical photosynthetically fixed organic carbon⁶, and the enzymes associated with chemosynthetic metabolism are present in their tissues^{7,8}. Here we report that fossil methane is the dominant source of carbon found in living tissues and recent authigenic carbonate minerals associated with abyssal brine seeps at the base of the Florida Escarpment. Most organic carbon and authigenic carbonates adjacent to the seeps contain progressively less modern carbon as their ^{13}C contents approach that of methane carried in the brine. Incorporation of fossil methane ($\leq 1.3\%$ modern carbon) into living tissues and carbonate cements results in recently formed materials which are depleted in ^{14}C . Therefore, ^{14}C cannot be used to indicate the age of authigenic materials produced at the pore-water-seepage environments that speckle the continental margins of the world.

The energy source for deep-sea chemosynthetic communities is reduced compounds, such as HS^- , CH_4 , NH_4^+ or petroleum^{9,10}, which may be carried in pore fluids from anoxic subsurface formations onto the oxygenated sea floor. Dissolved $\Sigma\text{H}_2\text{S}$ (H_2S , HS^- , S^{2-}), NH_4^+ and CH_4 are all present in brines that discharge onto the sea floor at the base of the Florida Escarpment^{11,12}. The extremely negative $\delta^{13}\text{C}$ values [$(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{Std}} \times 1,000$ ($< -70\%$ relative to the PDB standard)] found in tissues of animals living on patches of sediment enriched in isotopically light methane⁶ indicates the overriding importance of methylotrophy¹³. Biogenic methane is the only natural carbon source that has such depleted $\delta^{13}\text{C}$ values⁶. The chemosynthetic use of $\Sigma\text{H}_2\text{S}$ and NH_4^+ would require uptake of dissolved inorganic carbon which is enriched

in ^{13}C and therefore should produce isotopically 'heavy' organic tissue.

Earlier measurements of ^{14}C in animal tissues from this same site produced a surprising result; tissues with very negative $\delta^{13}\text{C}$ values contain significant amounts of modern carbon ($\sim 60\%$)⁶. Here we report new measurements of $\delta^{13}\text{C}$ and ^{14}C from the ambient methane, living animal tissues, bacterial mats, sedimentary organic carbon and authigenic carbonate carbon collected at the brine seeps during RV *ALVIN*'s 1986 visit to the Florida Escarpment (Table 1), which we use to establish the carbon sources and pathways.

The seep methane samples contain between 1.0% and 1.3% modern carbon. However, we cannot distinguish these measured values from 0% modern because they are close to the detection limit of the accelerator-mass-spectrometer technology used (0.4%) (ref. 14) and the extensive sample handling involved in concentrating the methane may have added to the ^{14}C background.

In our new, larger data set, the $\delta^{13}\text{C}$ and ^{14}C values of the animal tissues and other organic carbon samples exhibit two trends (Fig. 1). Data lying in envelope A (between ambient methane and average marine organic carbon) indicate that fossil methane mixed with varying amounts of pelagic organic matter is the dominant source of carbon in the seep organisms' tissues; some contain as little as 12.8% modern carbon. Other organic samples with extremely negative $\delta^{13}\text{C}$ values contain appreciable amounts of modern ^{14}C and exhibit a second mixing trend (B in Fig. 1). These data may indicate that there is a second source of young biogenic methane here⁶, generated by rapid methanogenesis using organic detritus in the sediments around the seeps. Methanogenesis in organic-rich sediments around the seeps should be stimulated by the nearly complete sulphate depletion, as it is in coastal sediments¹⁵. Such depletion results from mixing of pore fluids with sulphate-free seep fluids at the Florida Escarpment¹¹. Dual sulphide sources have been identified by stable-isotope analysis, some advected in with the brines and some produced by microbial sulphate reduction in the sediments¹¹. Thus, there may also be dual methane sources. A less likely, but possible explanation for the second mixing trend, is the contamination of samples by ^{14}C used in tracer experiments that are periodically conducted in shared laboratory facilities. We note that this trend is dominated by earlier measurements⁶.

The shells of living mussels contained $82 \pm 3\%$ modern carbon and a mean $\delta^{13}\text{C}$ (PDB) value of $-4.3 \pm 1.7\%$. These values are similar to those expected for bottom water¹⁶ and confirm that the dominant source of shell carbon is dissolved seawater bicarbonate.

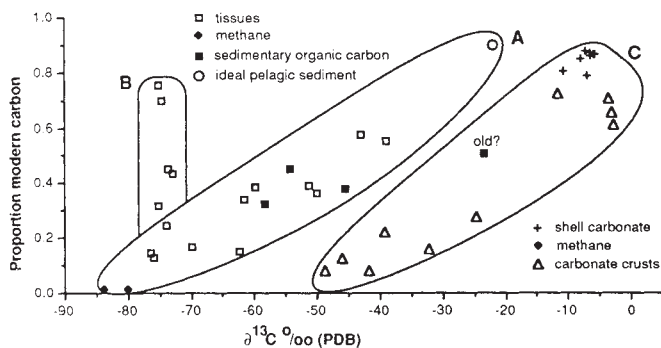


FIG. 1 Plot of the proportion of modern carbon and $\delta^{13}\text{C}$ values of carbon-carrying components associated with abyssal brine seeps at the base of the Florida Escarpment, Gulf of Mexico. Values are given in Table 1. Envelope A contains the organic carbon measurements and indicates the mixing trend between ideal planktonic organic carbon ($\delta^{13}\text{C}$ (PDB) = -22% and 90% modern carbon)²¹ and the ambient methane. Envelope B indicates a secondary trend in the organic data. Ageing causes one sedimentary sample to fall below the envelope. Envelope C contains the carbonate data.

TABLE 1 $\delta^{13}\text{C}$ and ^{14}C content of carbon in components of the seep environment

	Lab. no.	Organics		Lab. no.	Carbonates	
		$\delta^{13}\text{C}^*$	Modern C		$\delta^{13}\text{C}^*$	Modern C
Mussels						
1760-6 gill	AA3646	-70.0	0.1655 $\pm$ 0.004	AA3942	-3.5	0.8338 $\pm$ 0.005
1762-2 mantle	AA3653	-75.2	0.3174 $\pm$ 0.003	AA3945	-3.0	0.8351 $\pm$ 0.005
1762 gill	AA3647	-73.6	0.4509 $\pm$ 0.004	AA3945	-3.0	0.8351 $\pm$ 0.005
1763-5 gill	AA3966	-62.3	0.1489 $\pm$ 0.002	AA3943	-5.2	0.8211 $\pm$ 0.005
1763-1 gill	AA3650	-76.4	0.1427 $\pm$ 0.003	AA3944	-8.1	0.7795 $\pm$ 0.005
1764-10 mantle	AA3651	-76.0	0.1277 $\pm$ 0.003			
1769-2 mantle	AA3652	-74.0	0.2434 $\pm$ 0.003	AA3946	-2.7	0.8396 $\pm$ 0.005
1344-2†		-73.0	0.433		-4.3	0.848
1344-3†		-75.2	0.753		-4.1	0.762
1344-4†		-74.7	0.699		-3.5	0.842
Vestimentifera						
1764-4	AA3648	-50.0	0.3619 $\pm$ 0.003			
1770-3	AA3655	-39.0	0.5540 $\pm$ 0.004			
1771	AA3657	-51.3	0.3912 $\pm$ 0.003			
1343-A†		-43.0	0.581			
1343-B†		-42.0	0.576			
Surficial mat						
1770	AA3654	-59.9	0.3854 $\pm$ 0.003			
1771	AA3656	-61.6	0.3409 $\pm$ 0.003			
Methane						
1763	AA2305	-80.0	0.013 $\pm$ 0.002			
1764	AA2306	-83.7	0.010 $\pm$ 0.002			
1765	AA2304	-80.0	0.011 $\pm$ 0.002			
Sediment samples						
1343 scoop†	B28722	-45.4	0.382 $\pm$ 0.005	B28723	-22.9	0.263 $\pm$ 0.006
1765-1 0-6 cm				B26369	-0.4	0.680 $\pm$ 0.015
1765-1 18-22 cm				B26370	0.5	0.588 $\pm$ 0.009
1768-1C 0-10	B30307	-23.4	0.508 $\pm$ 0.005	B30308	0.1	0.628 $\pm$ 0.008
1769-4D 0-6 cm	B30912	-54.2	0.455 $\pm$ 0.013	B30310	-38.6	0.208 $\pm$ 0.003
1770-3A 0-6 cm	B30311	-58.4	0.337 $\pm$ 0.006	B30312	-9.0	0.694 $\pm$ 0.008
Crusts						
1760-1				B26368	-45.6	0.113 $\pm$ 0.008
1765-4B				B26371	-30.9	0.147 $\pm$ 0.004
1771-2				B26372	-48.8	0.068 $\pm$ 0.004
1770-3B				B28722	-41.2	0.070 $\pm$ 0.005

Ratios of $^{14}\text{C}/^{13}\text{C}$ from ref. 9 are indicated with AA laboratory numbers. We measured the ^{14}C content of carbonates (acid-volatile carbon) and disseminated organic carbon (combustible carbon after removal of acid-volatile carbon) in sediment samples by traditional β -counting at Beta Analytic, Miami, (B laboratory numbers). Samples include: tissues and shells of mussels and vestimentifera (*E. laminata*) (collected alive), surficial bacterial mats, methane, organic carbon and carbonate carbon from scoop samples, short cores and cemented crusts. Samples of ambient methane were collected with an *in situ* sampling device called Whimper; it extracts pore waters from the seep sediment and keeps them sealed to avoid degassing. White organic mats of bacterial origin which covered the sediment surface were collected with a 'floc-sucker', a sea-floor vacuum cleaner. Mussels, vestimentifera and carbonate crusts were collected from the sediment surface and other sediments were cored.

*Relative to the PDB standard.

† From ref. 6.

Carbonate in the background hemipelagic sediments of the abyssal Gulf of Mexico contains $69.0 \pm 2.9\%$ modern carbon ($\sim 3,068$ yr BP) (Table 1). Surficial sediments typically show age offsets associated with sediment bioturbation¹⁷. Bulk samples from the carbonate crusts contain carbon with $\delta^{13}\text{C}$ values well outside the range of normal marine carbonates ($\delta^{13}\text{C}$ (PDB) down to -48.8%) and ^{14}C contents that are very depleted (7% modern carbon) relative to other surficial material. Crusts contain variable mixtures of background pelagic carbonate, *in situ* shell hash, and carbonate cement⁵. We believe that the crusts are formed in recent times because: (1) the surface sediments adjacent to the seeps contain pelagic carbonate with Holocene ^{14}C concentrations (Table 1), (2) the cemented crusts contain *Globorotalia menardii*, a species of planktonic foraminifera that only occurs in the Atlantic during interglacials¹⁸, and (3) several hundred metres of sediment accumulated in this area of the abyssal Gulf of Mexico during the Pleistocene, indicating that Pleistocene material should be deeply buried¹⁹. A strong correlation exists between the $\delta^{13}\text{C}$ values and ^{14}C content ($r^2 = 0.87$) of all carbonate samples (envelope C in Fig. 1). The measurement of anomalously low $\delta^{13}\text{C}$ values and depleted ^{14}C suggests

that oxidized ambient methane is the carbon source for these cements. The spread in $\delta^{13}\text{C}$ values from the crusts reflects varying proportions of methane-derived cements and normal marine carbonates. Therefore ^{14}C measurements on surficial carbonate samples with unusually low $\delta^{13}\text{C}$ values²⁰ may reflect fossil methane sources. □

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Halide systematics of submarine hydrothermal vents

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CHLORIDE is the dominant anion in submarine hydrothermal fluids and plays a fundamental part in controlling their chemistry¹. The endmember Cl concentrations in virtually all submarine hot springs deviate from ambient sea water ($\sim 541 \text{ mmol kg}^{-1}$) and range from 188 mmol kg^{-1} at the ASHES vents on Axial Volcano² to $1,090 \text{ mmol kg}^{-1}$ at the Southern Juan de Fuca Ridge³. A variety of mechanisms have been proposed to explain these Cl enrichments and depletions including: rock hydration and dehydration⁴, phase separation^{2,3,5–7}, and the formation and dissolution of a retrograde soluble phase⁸. Support for the latter hypothesis came from hydrothermal fluid/basalt experiments which showed large changes in dissolved Cl at specific reaction conditions⁹ and also from the presence of Cl-rich minerals phases in ultramafic rocks¹⁰. We have carried out the first systematic study of Br and I in mid-ocean ridge hydrothermal vent fluids to constrain the models of halide diversity better by ascertaining their behaviour relative to Cl. The cohesiveness of the Br and Cl data and the similarity of Br/Cl to the seawater ratio suggests a single, non-fractionating process controls the salinity of sediment-starved mid-ocean ridge hydrothermal systems. The thermal decomposition of organic matter provides an additional source of Br and I in sediment-hosted systems.

We analysed the Br and I by inductively coupled plasma-mass spectrometry (ICP-MS), which uses a high-temperature argon plasma as an ion source for a quadrupole mass spectrometer. The results are presented in Tables 1 and 2 and the endmember Br are plotted against Cl concentrations in Fig. 1. (Fluoride is completely removed in high-temperature endmember fluids^{4,8} and will not be considered here.) The samples were obtained from a variety of geological settings including sediment-starved, mid-ocean-ridge (MOR) systems along the slow-rifting Mid-Atlantic Ridge (MAR)¹¹, fast- and intermediate-spreading portions of the East Pacific Rise (EPR)^{14,12}, the Southern Juan de Fuca (SJdF)³, the Marianas Trough Back-arc Basin¹³, and sediment-hosted systems in both the Guaymas Basin, Gulf of California¹² and the Escanaba Trough, Gorda Ridge¹⁴. Some regions have both Cl enrichments and depletions at different vents (21° N EPR (refs 4, 12), 11° N EPR (ref. 1) and Axial Volcano (ref. 2)), whereas other regions only have Cl enrichments [13° N EPR (refs 1, 15–16) and SJdF (ref. 3)]. Time-series studies have established that the endmember chemical compositions (including Cl) of both sediment-starved and sediment-hosted hydrothermal systems remain stable for many years¹². Therefore, the diversity in Cl concentrations at different vents cannot be attributed to transient phenomena and any model must explain the halide buffering effect.

The Br data for sediment-starved hydrothermal fluids are linear with respect to Cl (Fig. 1) and the Br/Cl values (Table 1) are scattered just above the seawater ratio ($1.55 \times 10^{-3} \pm 0.04$). The Br/Cl regression line has a slope of 1.68×10^{-3} and, within the 2σ error, extrapolates to the origin with a value for the

square of the correlation coefficient, r^2 , of 0.992. The slope of the Br/Cl line is similar to the value for Rekyjanes Ridge tholeiites¹⁷, but Br and Cl concentrations in basalts are too low for it to be an important source of halides. Mantle exhalations have also been suggested^{15,16} as a source of Br and Cl enrichments at 13° N EPR. Neither mantle degassing nor extraction of halides from basalts, however, can explain Cl-depleted fluids. The coherence of the Br and Cl data presented here suggests that their chemistry is controlled by a single process, which is responsible for the halide diversity at MOR systems.

Sediment-hosted hydrothermal systems in both the Guaymas Basin and the Escanaba Trough have significantly larger Br/Cl ratios than MOR systems (Fig. 1). Large amounts of I also occur in sediment-hosted fluids (Table 2) in contrast to sediment-starved fluids, where it was below the detection limit of our analysis ($\sim 1 \mu\text{mol kg}^{-1}$). The ratio of excess Br (relative to sediment-starved vents at similar chlorinities) to excess I is 1.4–2 at Guaymas and 1 at Escanaba (Table 2). Bromide and iodide are associated with organic matter in sediments¹⁸ and in unaltered Guaymas surface sediments $\text{Br/I} \approx 2$ (J. Gieskes and A. Magenheimer, personal communication). In the Guaymas Basin vents the I-content variations correlate with NH_4^+ (Fig. 2) and other chemical species derived from the thermal decomposition of organic matter in the sediment (A.C.C. *et al.*, manuscript in preparation). The relatively low NH_4^+ and high I content of the Escanaba fluids is consistent with the more oxidizing nature of these sediments compared to Guaymas. The fact that the Br-Cl trend for Guaymas and Escanaba is parallel to sediment-starved systems (Fig. 1) suggests that, in addition to fluid-sediment interactions, a process similar to that occurring at MOR systems also affects their halide chemistry.

The Br data from sediment-starved systems have important implications for models of Cl variability that invoke a retrograde soluble phase. The formation and dissolution of phases such as $\text{Fe}_2(\text{OH})_3\text{Cl}$ or magnesium hydroxylchlorides could explain⁸ the inter-field variations in salinity at the Galapagos vents. Hydrothermal fluid/basalt experiments⁹ seem to support this hypothesis: preferential uptake of Cl relative to Br from solution occurs during the initial, high-temperature phase of the reaction (425°C and 400 bars) and release of Cl occurs as the temperature is then decreased (250°C). The formation and subsequent dissol-

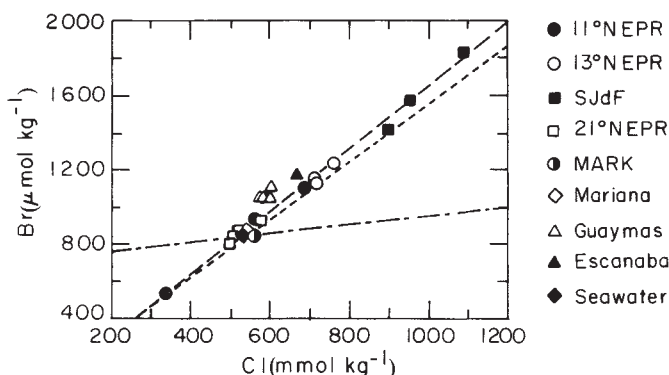


FIG. 1 Bromide plotted against chloride in endmember hydrothermal fluids. The Br/Cl slope for the sediment-starved vents analysed in this work (1.68×10^{-3}) is indicated by the long dashes and the seawater ratio (1.55×10^{-3}) is shown by the short dashes. The trend for the change in Br relative to Cl in hydrothermal fluid/basalt experiments⁹ is shown by the short-long dashes. For the ICP-MS analyses we used 1:100 dilutions of hydrothermal fluids in 0.5% (v/v) HNO_3 or HCl . We determined the accuracy of the Br analyses by measuring 11 ambient sea water and IAPSO (International Association for the Physical Sciences of the Ocean) standard seawater samples during different analytical runs. The mean value of $840 \pm 23 \mu\text{mol kg}^{-1}$ compares favourably with the accepted range for sea water³⁰. Duplicate analyses of 14 vent-fluid samples during different analytical runs gave a precision of $\pm 26 \mu\text{mol kg}^{-1}$. Calibration of the ICP-MS method with a titration method³¹ produced agreement to better than $\pm 2\%$.

ution of $\text{Fe}_2(\text{OH})_3\text{Cl}$ was proposed to be responsible for this phenomena. If Cl-depleted hydrothermal fluids are generated by processes similar to those occurring in the experiment, then the Br/Cl ratio should be higher than sea water. Fluids enriched in Cl should display a decrease in Br/Cl compared to sea water, because the dissolving phase is expected to be depleted in Br relative to Cl. Figure 1 shows that the hydrothermal fluids do not follow the predicted variation in Br/Cl extrapolated from the experimental data.⁹ These Br/Cl results do not eliminate a retrograde soluble phase as the source of halide diversity, but impose the constraint that no significant fractionation between Br and Cl can occur during the formation or dissolution of such a phase. This seems unlikely for $\text{Fe}_2(\text{OH})_3\text{Cl}$, however, given the differences in crystal chemistry of the Cl and Br analogues.¹⁹

The presence of $\text{Fe}_2(\text{OH})_3\text{Cl}$ in oceanic rocks was hypothesized to explain the apparent Fe:Cl ratio observed in electron-microprobe studies of fine-grained serpentine^{10,20,21}. More-crystalline serpentine contains no Cl and very little Fe. The conversion of olivine and orthopyroxene to serpentine apparently occurs through this fine-grained intermediate phase²⁰. Its initial formation and subsequent recrystallization could act as a halide buffer for serpentinizing fluids. Although the data are limited, serpentines do show both Br and Cl enrichments²² and so the process may occur without large fractionation of Br and Cl.

Gabbros also contain Cl-rich phases, such as amphibole and

apatite, which are thought to originate from brines formed by phase separation²³⁻²⁵. If processes that produce Cl-rich phases in either peridotites or gabbros affect the halide content of MOR vent fluids then circulation cells operating within the deep oceanic crust must be coupled to shallower circulation in layer II basalts at the ridge axis. Oxygen isotope studies of ophiolites do indeed show seawater penetration into gabbros and peridotites, but the proposed shape of the magma chamber precludes coupling of these deeper circulation systems with shallower ones, except at considerable distance from the ridge axis²⁶. In contrast to this view a circulation model for the Kuroko ore deposits indicates that, under some circumstances, fluid flow in layer III gabbros and layer II basalts may be interconnected²⁷.

Phase separation is frequently invoked to explain halide variability of vent fluids^{2-3,5-7}. But, there are many aspects of the fluid chemistry spanning a wide range in Cl concentrations that are inconsistent with this hypothesis¹. Only the relatively shallow ASHES vents (~1,500 m), where the two-phase boundary is ~40–90 °C lower in temperature than for deeper (2,500–3,800 m) MOR systems⁵, show characteristics of phase separation, such as enrichment of volatiles in the low-salinity fluids². These solutions also display significant fractionation of Br from Cl ($\text{Br/Cl} = 1.28 \times 10^{-3}$; ref. 2), and thus are not a relevant model for the deeper MOR systems. The high salinities and apparent dissolved-gas depletions of the Southern Juan de Fuca fluids

TABLE 1 Bromide and chloride in sediment-starved vents

Region	Vent	Br ($\mu\text{mol kg}^{-1}$)	Cl (mmol kg^{-1})	Br/Cl ($\times 10^3$)
11° N EPR	6	533 ± 41	338	1.58 ± 0.12
11° N EPR	5	1,105 ± 17	686	1.61 ± 0.02
11° N EPR	4	940 ± 19	563	1.67 ± 0.03
13° N EPR	3	1,242 ± 59	760	1.63 ± 0.08
13° N EPR	2	1,158 ± 16	712	1.63 ± 0.02
13° N EPR	1	1,131 ± 62	718	1.58 ± 0.09
13° N EPR	N & S sites*	1,163	740	1.57
13° N EPR	Chandelier†	1,270	882	1.44
21° N EPR	OBS	802 ± 35	500	1.60 ± 0.07
21° N EPR	SW	877 ± 30	525	1.67 ± 0.06
21° N EPR	HG	855 ± 28	506	1.69 ± 0.06
21° N EPR	NGS	929 ± 34	579	1.66 ± 0.04
S. JdF	Plume	1,832 ± 44	1,090	1.68 ± 0.04
S. JdF	Vent 3	1,580 ± 35	951	1.64 ± 0.04
S. JdF	Vent 1	1,422 ± 175	896	1.59 ± 0.20
Mid-Atlantic Ridge	MARK	847 ± 12	559	1.52 ± 0.02
Mariana Trough		866 ± 19	544	1.59 ± 0.02
Sea water		840 ± 23	542	1.55 ± 0.04

The Br data for each vent (this work) are mean endmember values ± the standard deviation calculated from two or more samples. Most of the samples are greater than 96% hydrothermal fluid (assuming that any Mg in the sample is due to seawater entrainment during collection or near-surface mixing⁴). Samples from Southern Juan de Fuca and Mariana Trough were more diluted with ambient sea water. Iodide was below the detection limit for these ICP-MS analyses ($<1 \mu\text{mol kg}^{-1}$ in the undiluted sample). Sources of Cl data: 11–13° N EPR, vents 1–6 from ref. 1; 21° N EPR from ref. 12; S. JdF from ref. 3; Mid-Atlantic Ridge, MARK from ref. 11; Mariana Trough from ref. 13.

* Source of Br and Cl for 13° N EPR, N and S sites from ref. 15.

† Source of Br and Cl for 13° N EPR, Chandelier from ref. 16.

TABLE 2 Iodide, bromide and chloride in sediment-hosted vents

Region	Vent	I ($\mu\text{mol kg}^{-1}$)	Br ($\mu\text{mol kg}^{-1}$)	Cl (mmol kg^{-1})	Br/Cl ($\times 10^3$)	$\Delta\text{Br}/\Delta\text{I}^*$
Guaymas	E. Hill	58 ± 0.3	1,063 ± 21	603	1.76 ± 0.03	1.5
Guaymas	S. Field-1	70 ± 5	1,117 ± 38	604	1.85 ± 0.06	2.0
Guaymas	S. Field-2	76 ± 1	1,056 ± 76	587	1.80 ± 0.13	1.4
Guaymas	S. Field-3	77	1,054 ± 83	581	1.81 ± 0.14	1.4
Escanaba		87 ± 2	1,179 ± 23	668	1.76 ± 0.03	1.0

Sources of Cl data: Guaymas from ref. 12; Escanaba from ref. 14.

* Excess Br (ΔBr) in sediment-hosted vent fluids calculated as difference between Br/Cl for vent and mean Br/Cl for sediment-starved vents ($1.62 \pm 0.05 \times 10^{-3}$) times Cl concentration; excess I (ΔI) calculated as vent concentration minus seawater value ($\sim 0.4 \mu\text{mol kg}^{-1}$).

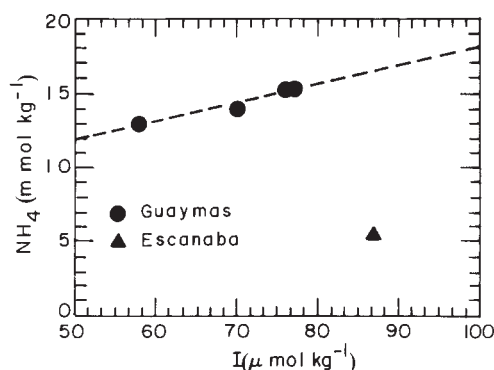


FIG. 2 Total iodide plotted against ammonium for sediment-hosted hydrothermal vents in the Guaymas Basin and Escanaba Trough. The correlation line for the Guaymas vents is shown by the dashes ($r^2=0.96$). The I/N ratio is consistent with the extraction of these components from organic matter in the sediments. The precision of the iodide analyses was $\sim \pm 3\%$.

may be due to the mixing of brine (formed by previous phase separation) with hydrothermal fluids having salinities roughly that of sea water³. This requires⁶ that Cl-rich fluids have larger $\delta^{18}\text{O}$ values than Cl-depleted fluids, because of fractionation effects, but no consistent trends with respect to Cl are observed for the Southern Juan de Fuca fluids²⁸. Because of the complexity of oxygen isotope systematics²⁷⁻²⁹ and the lack of experimental vapour-brine fractionation factors at appropriate temperatures and pressures, however, this problem cannot be resolved on the basis of the ^{18}O data alone.

Our systematic study of Br and I in submarine hydrothermal vents provides important constraints on models of halide diversity. In sediment-starved MOR vents the similarity of Br/Cl to sea water over a wide range in Cl concentrations reveals that the process controlling the salinity occurs without fractionation of Br relative to Cl. Sediment-hosted vents show enrichments in Br and I derived from the thermal decomposition of organic matter in the local sediments. Discrepancies between proposed models and observations must be resolved before the mechanism controlling salinity is elucidated. □

Contribution of induced magnetization to magnetization of seamounts

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A FUNDAMENTAL assumption in modelling seamount magnetic anomalies is that the contribution of induced magnetization is negligible. The general consistency of seamount and non-seamount palaeopoles, scarcity of poles skewed toward the present field direction and the high ratio of remanent to induced magnetization (Koenigsberger ratio) of many oceanic basalts have been cited as evidence supporting this assumption^{1,2}. Recent discussions concerning the dominance of normally magnetized seamounts have focused attention on the possible role of viscous and induced magnetization in seamount anomalies³⁻⁶. Here we determine natural remanent magnetization, initial volume susceptibility and the Koenigsberger ratio for more than 2,000 samples from a subaerially exposed seamount section on La Palma, Canary Islands (Table 1). By contrast to results from the oceanic crust and ophiolites, these data indicate that a variety of rock types are potential magnetic sources. The significant induced component of intrusives underscores the importance of the lithological distribution in determining the character of seamount magnetic anomalies. The La Palma data, together with a plausible lithological distribution, indicate that induced magnetization may account for one-sixth of seamount magnetization.

Previous measurements of the magnetic properties of seamount rocks are highly variable and are insufficient to determine adequately the relative contribution of induced and remanent magnetization to seamount magnetic anomalies. For example, pillow basalts dredged from seamounts have high Koenigsberger (Q) ratios^{7,8} indicative of negligible induced magnetization (IM), whereas drillcore samples from seamounts and islands yield moderate to low Q ratios⁹⁻¹¹. Low Koenigsberger ratios in gabbroic xenoliths from Jasper Seamount also indicate a large IM contribution from coarse-grained material in seamounts⁸. The variety of magnetic behaviour observed from this limited number of studies suggests that a comprehensive investigation of the magnetic properties of various seamount lithologies is warranted.

In the absence of further drilling into seamounts, subaerial exposures provide the best opportunity to characterize the magnetic properties of seamount lithologies. The Pliocene Seamount Series of La Palma comprises a 6-km uplifted and tilted sequence of alkalic seamount extrusives and intrusives that is unconformably overlain by subaerial flows of the Coberta Series¹². The Seamount Series consists of (1) a basal plutonic complex, (2) a locally sheeted dyke/sill complex, and (3) a 1,700-m section of bedded submarine pillows, hyaloclastites and breccias. Hydrothermal alteration of the Seamount Series is recorded by zeolite facies assemblages in the upper extrusive series, increasing to greenschist assemblages in the lower part of the section.

Natural remanent magnetization (NRM) directions of La Palma extrusives are dominantly of normal polarity although intrusives contain a significant reversely magnetized population. Demagnetization curves reveal low-stability components in many samples which often are large enough to obscure the original polarity. These data indicate further complications in the use of seamount magnetic anomalies; however, we focus here on the lithological dependence and relative importance of remanent and induced magnetization.

NRM intensities for all lithologies are well approximated by log normal or superposed log normal distributions (Fig. 1). We have listed both the geometric mean (GM), appropriate for log normal distributions, and the arithmetic mean (AM) of our data

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one-third to one-half induced magnetization. Correspondingly low Q ratios in dykes and gabbros have been reported from ophiolites, dredged and drilled material¹³⁻¹⁵. The induced magnetization in subaerial basalts ($Q = 18.69$) is less than for dredged seamount basalts^{7,8}, but higher than values reported for basalts from the Emperor Seamounts^{9,11}. The effect of metamorphism is to increase the relative contribution of induced magnetization, as indicated by the lower Q ratios in epidotized pillows (3.69). This effect has been noted in ophiolites¹⁹ and is consistent with the downhole decrease of Q in DSDP Hole 504B (ref. 15).

The lithological dependence of magnetic properties in samples from La Palma indicates that the distribution of lithologies within seamounts may have a key role in determining the character of the observed anomaly. The distribution of lithologies within seamounts is not well known, but several observations suggest that intrusives may constitute a significant volumetric portion of seamounts. Seismic evidence in conjunction with gravity modelling indicates that substantial amounts of intrusives occur within Hawaii, particularly beneath rift zones and extending up to shallow levels beneath the summit²⁰. The presence of dense feeder pipes within large seamounts and guyots is frequently inferred from inversions of gravity data²¹. Subaerial exposures on La Palma¹² and recent drilling results from Reunion Island^{22,23} also suggest that significant proportions of gabbroic and dyke material occur, at least within the central portion of oceanic volcanoes. Submersible and Deep Tow studies indicate that the flanks of oceanic islands are composed of pillows, volcanoclastic material and subaerially erupted flows²⁴. Seismic tomography of Jasper Seamount²⁵ suggests that smaller oceanic volcanoes may have a smaller proportion of intrusive material than large oceanic islands.

To estimate the potential contribution of induced magnetization, we constructed a model seamount whose lithological distribution is consistent with these observations (Fig. 3). The model is based on a radial profile (Profile 6) of Mauna Loa²⁶

and other assumptions given in the figure caption. The resulting model seamount contains 29% dykes, 14% gabbros, 22% pillows, 11% subaerial flows and 24% volcanoclastic material. Using the average values from La Palma gives a mean remanent magnetization of 4.90 A m^{-1} and an induced magnetization of 0.98 A m^{-1} . The remanent contribution resides primarily in the subaerial flows, and to a lesser extent in gabbros and pillows, whereas the IM is dominantly associated with the plutonic core (Fig. 3). The magnitude of IM (17% of the total magnetization) inferred from the La Palma data is consistent with the combined induced and viscous contribution deduced from model intensities of normally and reversely magnetized seamounts⁶.

The lithological distribution in Fig. 3 should be most applicable to large Hawaiian-type volcanoes and the effects of IM most pronounced in the older volcanoes of the Hawaiian-Emperor chain. Anomaly modelling and palaeomagnetic samples from Suiko Seamount (44.5° N) provide an opportunity to evaluate this model. Although palaeomagnetic samples from Suiko have reversed, high-stability components corresponding to a palaeolatitude of 27° N (ref. 26), only models involving the upper 10% of the volcanic edifice give comparable palaeolatitudes²⁷. Models that include the bulk of the seamount (such as model 4 in ref. 27) give far-sided palaeopoles and low intensities. These shallow-model inclinations²⁷ are consistent with a significant ($\sim 1/3$) induced and/or viscous magnetization and a larger uniform remanence acquired during construction of the volcano at 19.5° N , the present location of the Hawaiian hotspot.

The magnetic properties of samples from La Palma and a reasonable lithological distribution suggest that IM may constitute a significant portion of seamount magnetization; however, the predicted bias in seamount palaeopole positions is not readily apparent². This discrepancy may result from (1) differences between the magnetic properties of the La Palma sample collection and *in situ* seamount rocks, (2) smaller proportions of intrusive material, or (3) a bias in the selection of seamount anomalies that are modelled. Preferential selection of seamounts

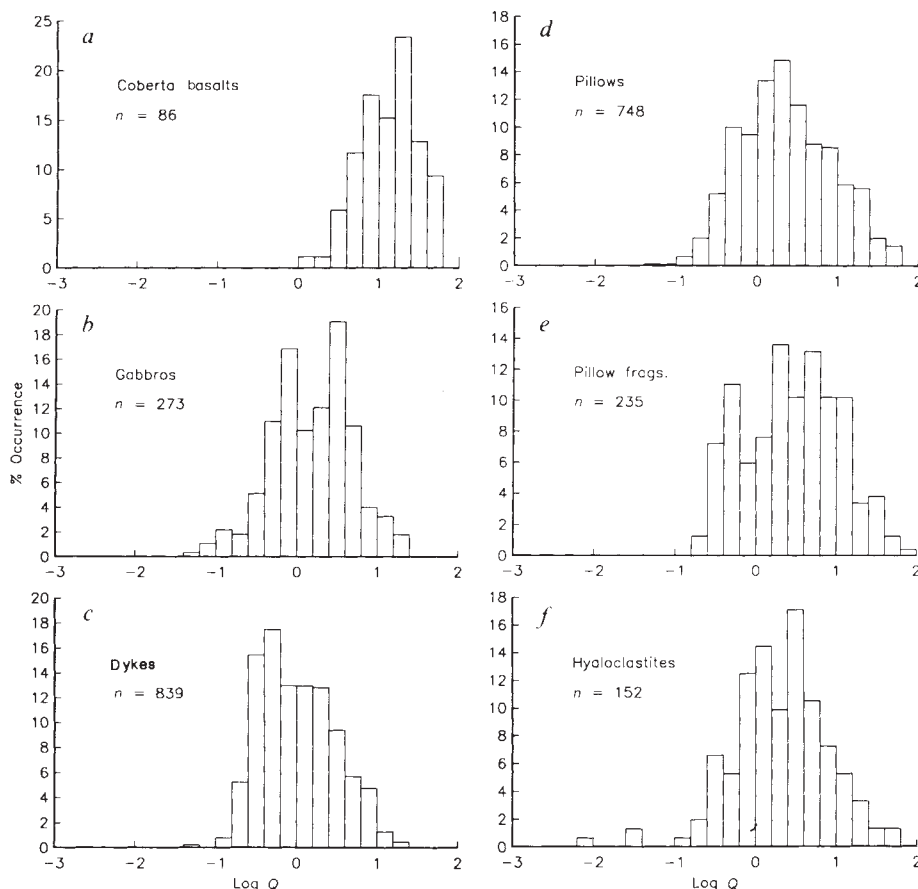


FIG. 2 Koenigsberger ratios of La Palma lithologies. Note the low Q values of intrusive material. Mafic gabbros have significantly higher Q than their more felsic counterparts (see Table 1).

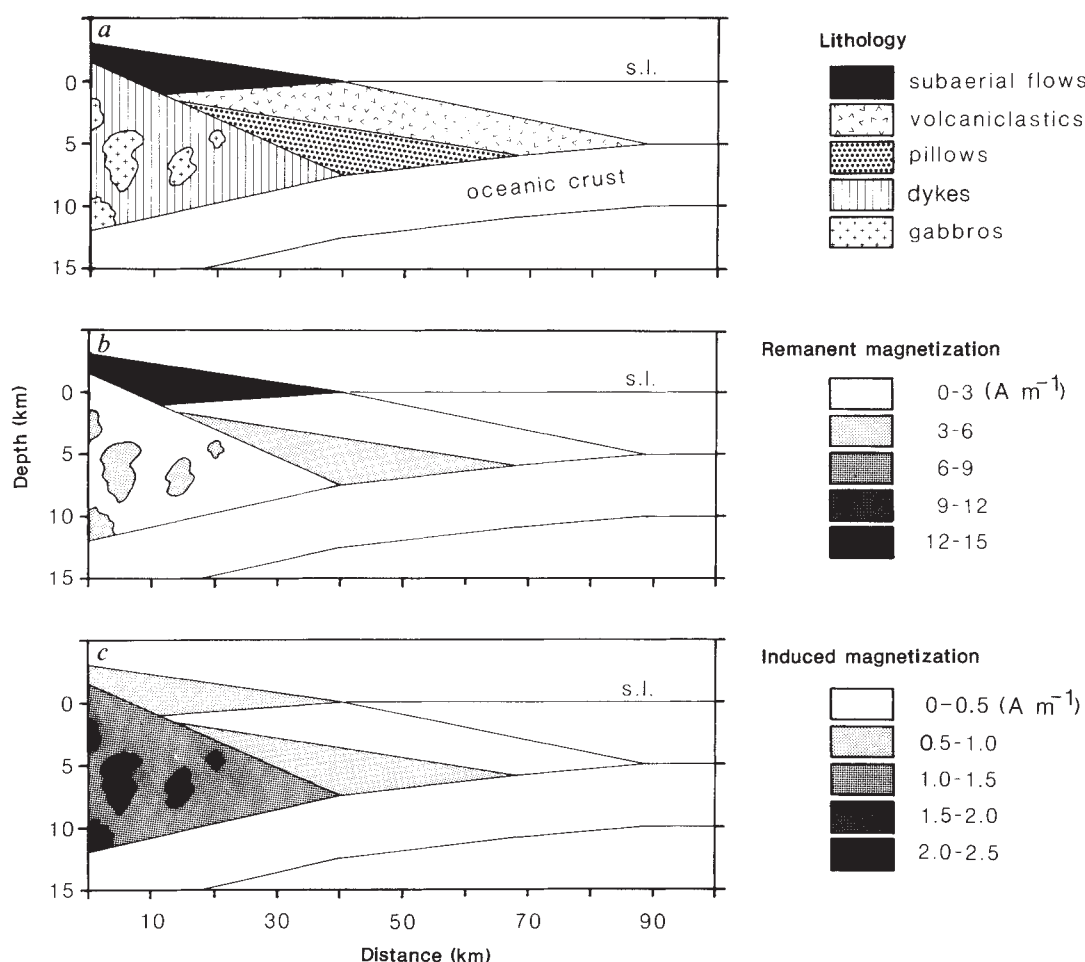


FIG. 3 *a*, Model lithological distribution and corresponding distributions of remanent (*b*) and induced (*c*) magnetizations. The lithological model is largely based on a radial gravity and seismic profile from Hawaii²⁰. We have also assumed that the high-density core is composed of 2/3 dykes and 1/3 gabbros. This is consistent with the 7.1 km s^{-1} velocity of this zone that implies the presence of some material more mafic than basaltic dykes²⁰. Pillow basalts are assumed to be similar to the subgreenschist pillows from La Palma. The outer, low-velocity layer has been divided into a subaerial portion (constrained by the present shoreline and a 500-m thick volcanoclas-

tic layer overlying the wedge of pillow basalts) and a volcanoclastic flank. Based on the lithological distribution in La Palma¹² and DSDP Sites 417 and 418 (ref. 29) the volcanoclastic zone is taken to be composed of 70% clastics and 30% pillows and the pillow zone 80% pillows and 20% clastic material. The resulting model seamount contains 29% dykes, 14% gabbros, 22% pillows, 11% subaerial flows and 24% volcanoclastic material. Using the AM (GM) from Table 1 gives an induced magnetization, concentrated in the intrusive core, which accounts for 17% (25%) of the total magnetization.

with simple magnetic-anomaly patterns and the under-representation of seamounts with inconsistent palaeopoles in the published literature may partially account for this discrepancy. As has been previously suggested⁸, small oceanic volcanoes that may be dominantly composed of extrusives should provide the best palaeopoles for plate reconstructions. Because the induced and viscous components are predominantly associated with intrusive material, we propose that the average density of seamounts may provide a useful additional discriminant for magnetic modelling. Seamounts with average densities greater than that of typical submarine extrusives (2.7 g cm^{-3}) (ref. 20) probably contain a significant portion of intrusives and may exhibit a correspondingly large induced contribution. □

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A comparison of energetics and ventilation of desert ants during voluntary and forced locomotion

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THE energy cost of pedestrian locomotion in terrestrial vertebrates has been extensively studied¹⁻³. Insects differ fundamentally from vertebrates, making data on their locomotion energetics of great comparative interest, but their small size and low behavioural plasticity complicate measurements. Miniature treadmills⁴⁻⁹ enforce unnatural running regimes; therefore no data exist on the energetics of insects running voluntarily, or on insect ventilation while running. We have now measured the minimum cost of transport and ventilation of the harvester ant *Pogonomyrmex rugosus* (mass, 17 mg) by using both treadmill and voluntary running. The minimum cost of transport was constant over temperature (34–43 °C) and lower than predicted from vertebrate data. Treadmill running significantly affected energetics, eliminating the slow ventilation (1–2 min⁻¹) in voluntarily running ants and substantially elevating extrapolated metabolic rate at zero running speed, which equalled the standard metabolic rate in voluntarily running ants.

Any complete description of an organism must include an account of its energy transactions. Of the ways animals expend energy, locomotion is usually of cardinal importance. The simplest measure of the cost of transport (COT) is obtained by dividing the mass-specific metabolic rate while running (MR) by the running speed, yielding the gross cost of transport (GCOT, for example, in J kg⁻¹ m⁻¹). But GCOT is speed-dependent, and a better comparative measure is the minimum cost of transport (MCOT), which is the slope of the generally linear relation between MR and speed¹. GCOT and MCOT share identical units, but MCOT is independent of speed and standard metabolic rate (SMR), allowing cross-comparisons between, for example, mammals and birds (high SMR), and reptiles and insects (low SMR)^{3,6,7}.

In terrestrial vertebrates, MCOT scales inversely across several orders of magnitude of body mass¹⁻³, with smaller animals having higher MCOT; the theoretical basis of this allometry is obscure. Terrestrial vertebrates, however, are physiologically an homogeneous group—all have internal skeletons and four or fewer walking limbs, and almost all ventilate tidally. Do insects, with exoskeletons, six walking limbs, low body mass, and putatively diffusion-based ventilation, scale similarly? Despite the technical problem of measuring MCOT in tiny animals, several investigators have published data on the subject, generally obtained by employing miniature treadmills of various designs⁴⁻⁹. These treadmills had high internal volumes that distorted or eliminated ventilation data. They also forced an animal with low behavioural plasticity to run at fixed speeds, maintaining its position on a moving belt by means of continuous feedback from unnaturally stationary surroundings. Therefore, no information on the ventilation of any insect during pedestrian locomotion exists, and it is not known how treadmill running affects COT relative to voluntary running. Lack of data on ventilation is particularly unfortunate, because ventilation in some adult insects is convective and discontinuous rather than purely diffusive and continuous¹⁰⁻¹², probably to reduce respiratory water loss^{12,13}. If insects can maintain discontinuous ventilation during activity, the loss of water from respiratory surfaces may be greatly reduced. The arid-adapted, ecologically important seed-harvester ant *Pogonomyrmex rugosus* was therefore a

logical choice of animal to study. And, because active foraging is the only means of energy acquisition for *P. rugosus*, COT parameters are of obvious and crucial significance in understanding and modelling its foraging ecology and the economic basis of its colonies.

Individual *P. rugosus*, enclosed within a metre-long tube of 10 mm internal diameter, ran back and forth voluntarily for >30 min at mean speeds that remained approximately constant for periods >5 min at a time. Locomotion was not appreciably affected by the tube, within which an ant could run and turn freely. Exploiting this behaviour, we designed a 'running-tube respirometer' in which the running speed and CO₂ emission of an individual ant could be monitored continuously (Fig. 1). Individual variation in running speeds was sufficient to determine MCOT accurately at four equally spaced, ecologically realistic¹⁴ temperatures between 34 and 43 °C, at which we also ran the ants using a miniature treadmill⁸ (Fig. 2).

MCOT, a measure of the incremental energy required to transport a unit of animal mass a unit distance, was equivalent in running-tube and treadmill runs at 188.2 J kg⁻¹ m⁻¹. We confirm previous findings for iguanid lizards¹⁵ and cockroaches⁵ that MCOT is independent of temperature (Fig. 2). Our value is, however, some 50% lower than that which current equations predict for an insect of equivalent body mass^{2,7}. During voluntary running, extrapolated MR at zero running speed (*y*-axis intercepts of graphs in Fig. 2) was equivalent to predicted SMR (ref. 16) at all temperatures. During treadmill running, the intercepts at all temperatures were significantly ($P < 0.001$) and substantially (72%) elevated above SMR. A *y*-axis intercept elevation above SMR is generally found in treadmill COT studies^{1,3,6,8} and is often regarded as a normal component of locomotory energetics referred to as 'postural cost'¹. Our data indicate that, in *P. rugosus* at least, the 'normal' *y*-axis-intercept

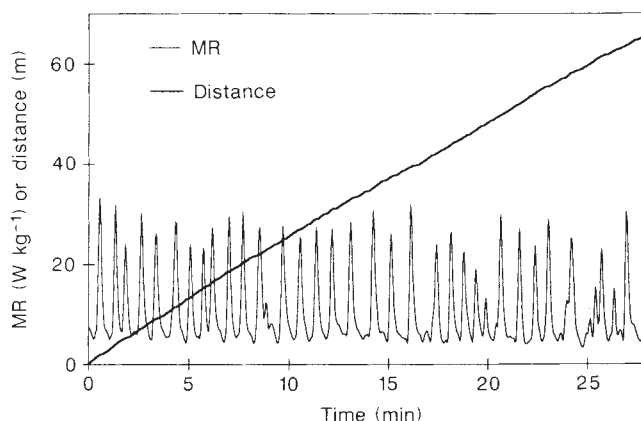
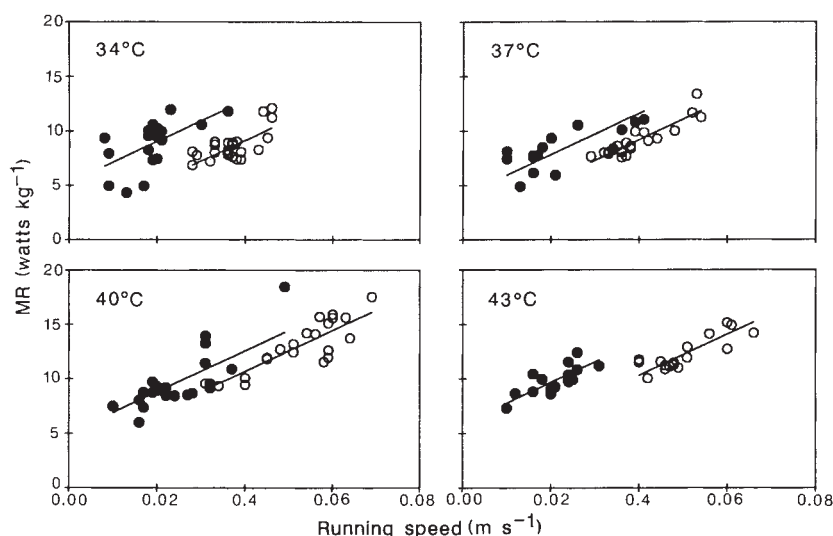


FIG. 1 Metabolic rate (MR, from CO₂ production) of a voluntarily running 16.5-mg *P. rugosus* ant at 40 °C (light trace), and total distance run (heavy trace). Mean speed from 0–15 min was 0.042 ± 0.003 m s⁻¹; from 15–25 min, 0.037 ± 0.003 m s⁻¹. Note that discontinuous ventilation causes cyclic variation in measured MR; actual MR is approximately constant over each cycle.

METHODS. Ants were enclosed in 1 m of tubing (10-mm i.d., Tygon) graduated at 10-mm intervals, temperature controlled to 0.05 °C and connected to a flow-through respirometry system^{8,12} (flow rate, 100 ml min⁻¹), or run at defined speeds with a treadmill⁸ modified to contain a dead volume of only 10 cm³ for fast response to ventilation, using an identical respirometry system. During each experiment, CO₂ production rate (VCO₂), temperature and ant speed were continuously monitored (Datacan IV, Sable Systems, Los Angeles, California). VCO₂ was converted to MR from the respiratory quotient (RQ) of active ants assuming fat-based metabolism. Active RQ measured in a closed system^{8,17} (0.68 ± 0.05) was similar to fat-based metabolism RQ of 0.71, yielding the energy equivalent 27.9 J cm⁻³ CO₂.

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FIG. 2 MR of running *P. rugosus* at four temperatures; open circles are running-tube data, closed circles are treadmill data ($N=30$ and 27 ants, respectively). Slopes of all lines do not differ (analysis of covariance, variance ratio; $F=1.1$; $P=0.35$; common slope ($\pm$ s.e.m.)= $188.2 \pm 12.9 \text{ J kg}^{-1} \text{ m}^{-1}$). For voluntary locomotion, y-axis intercepts do not differ from predicted SMR¹⁶ at any temperature ($P>0.1$, multiple comparison analysis of variance) but all treadmill locomotion intercepts are substantially higher than SMR (mean 72%; $P<0.001$). All regressions are significant at $P<0.001$.



elevation above SMR could be an artefact of an unnatural running regime. This contention is strengthened by our ventilation data. Ventilation was prominent during voluntary running (Fig. 1) at a low frequency ($\sim 1\text{--}2 \text{ min}^{-1}$) strongly correlated with MR and hence running speed ($P<0.0001$). By contrast, treadmill running frequently abolished discontinuous ventilation altogether and, when it occurred, it was faster ($\geq 2 \text{ min}^{-1}$) and did not change with MR or temperature. Treadmill running therefore profoundly affected the animals' respiratory responses to exercise.

The MCOT/body-mass allometry of insects and vertebrates cannot yet be meaningfully compared because the insect data are too variable; currently, body mass accounts for $<45\%$ of MCOT variation (data from refs 5–8, 17). In the light of our findings, voluntary running regimes, applied across a wide range of body masses, should yield more consistent and reliable data on insect MCOT/body-mass allometry.

Contrary to the conventional diffusion model of non-flight insect ventilation¹⁸, *P. rugosus* ventilated discontinuously, even at high running speeds and temperatures. The very low frequency of this ventilation, combined with the small size of *P. rugosus* and its high level of activity at high temperatures, indicate the possibility of exceptional resistance to respiratory water loss relative to ventilation based solely on diffusion. The extent of discontinuous ventilation during activity in other arid- or mesic-adapted insects remains unknown. □

Subthreshold Na^+ -dependent theta-like rhythmicity in stellate cells of entorhinal cortex layer II

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THE oscillation of membrane potential in mammalian central neurons is of interest because it relates to the role of oscillations in brain function. It has been proposed that the entorhinal cortex (EC), particularly the stellate cells of layer II (ECIIscs), plays an important part in the genesis of the theta rhythm^{1–3}. These neurons occupy a key position in the neocortex–hippocampus–neocortex circuit, a crucial crossroad in memory functions^{4,5}. Neuronal oscillations typically rely on the activation of voltage-dependent Ca^{2+} conductances and the Ca^{2+} -dependent K^+ conductance that usually follows^{6,7}, as seen in other limbic subcortical structures generating theta rhythmicity^{8–10}. Here we report, however, that similar oscillations are generated in ECIIscs by a Na^+ conductance. The finding of a subthreshold, voltage-gated, Na^+ -dependent rhythmic membrane oscillation in mammalian neurons indicates that rhythmicity in heterogeneous neuronal networks may be supported by different sets of intrinsic ionic mechanisms in each of the neuronal elements involved.

The EC, by way of the 'perforant path', is the chief source of afferents to the hippocampus¹¹. The main component of this projection originates from ECIIscs^{12,13}, which activate the hippocampal 'trisynaptic circuit'^{14,15}. Theta activity in this system is believed to underlie synaptic plasticity^{16,17}. Our results indicate that this rhythmicity is an intrinsic property of the cells driving the neuronal chain.

Stellate cells constitute the principal cell type of layer II of the EC (ref. 11). They have numerous primary dendrites which branch repeatedly as they distribute over layers I and II (Fig. 1a); their descending axons (a) give off recurrent collaterals.

The electrophysiological characteristics illustrated in Fig. 1b were seen in 63 oscillatory cells of 81 neurons recorded in layer

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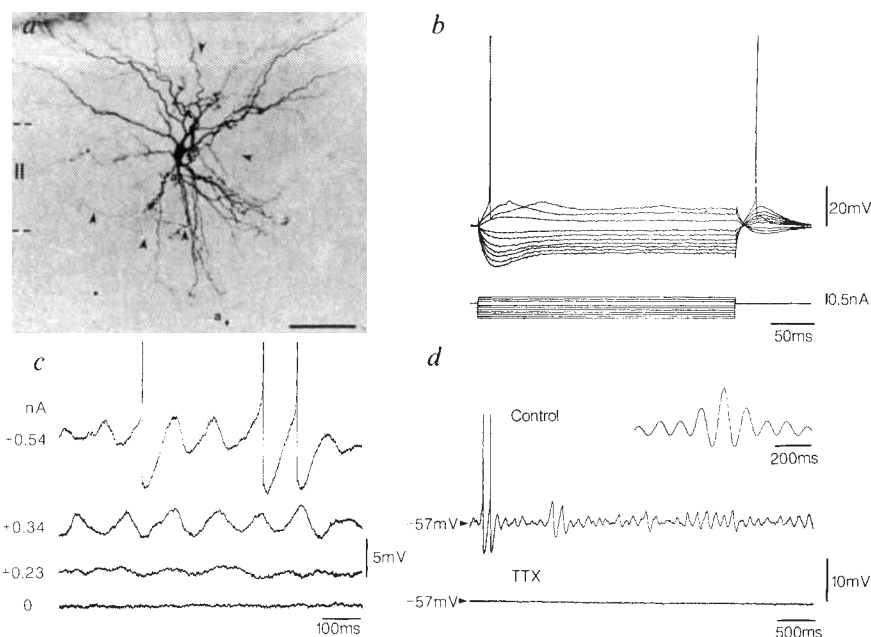
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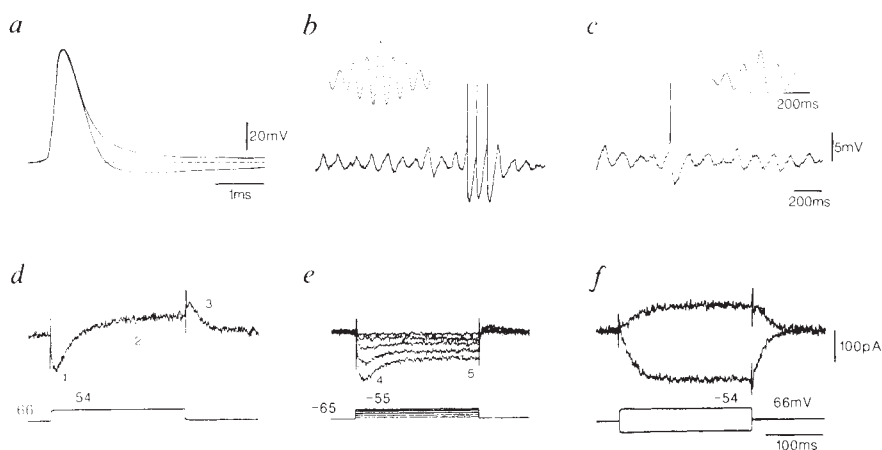
FIG. 1 Morphological and electrophysiological properties of ECIIs. *a*, Photomicrograph of a biocytin injected ECIIs (revealed with avidin-biotin-peroxidase complex²⁷). The axon (*a*) gives off several recurrent collaterals (arrowheads). The pial surface is at the top and limits of layer II are indicated (broken lines). Scale bar, 100 μ m. The electrophysiological properties of the cell in *a* are illustrated in *b-d*. *b*, Delayed and anomalous rectifications are evident. Note that the membrane potential overshoots the resting potential (-64 mV) after the break of the inward current pulses. *c*, Subthreshold voltage oscillations for three levels of depolarizing constant current injection from rest. The oscillatory activity became most apparent with current injection of $+0.34$ nA (mean membrane potential, ~ -57 mV). *d*, The auto-correlogram (inset) illustrates the rhythmic character of the voltage oscillation at a dominant frequency of 8 Hz. Note that this oscillatory activity was abolished by bath application of TTX (1μ M) (bottom trace).

METHODS. By using standard procedures²¹, 400 μ m horizontal brain slices were made from the retrohippocampal region of the adult rat. Slices were submerged at $35 \pm 1^\circ\text{C}$ and superfused with a solution containing (in mM): NaCl, 124; KCl, 5; KH_2PO_4 , 1.2; MgSO_4 , 2.6; CaCl_2 , 2.4; NaHCO_3 , 26; glucose, 10 (saturated with 95% O_2 and 5% CO_2). Neurons in layer II of the medial EC were impaled with microelectrodes filled with 3–4 M potassium acetate (40–90 M Ω) or 2% biocytin in 2 M potassium acetate for intracellular labelling. In some experiments microelectrodes filled with 3 M potassium acetate with 15–100 mM QX314 were used. Recordings were made with an Axoclamp II amplifier in either the conventional bridge-current clamp (Fig. 1; Fig. 2*a-c*) or the single-electrode voltage-clamp mode (Fig. 2*d-e*). In the



latter case the head-stage voltage was monitored to ensure complete settling during each switching cycle (frequency, 3–5 kHz). When Co^{2+} and Cd^{2+} were used, phosphate and sulphate were omitted to prevent precipitation. The divalent cations were maintained at a constant concentration by adjusting the Mg^{2+} concentration. Slices containing biocytin-labelled neurons were re-sectioned at 50 μ m and processed using the avidin-biotin-peroxidase complex²⁷.

FIG. 2 Ionic basis of the subthreshold membrane-potential oscillations in ECIIs. *a*, Superimposed spikes before and during 10 min perfusion with a medium containing lowered Ca^{2+} (1.5 mM), 0.3 mM Cd^{2+} and 2 mM Co^{2+} . Note the pronounced broadening of the base of the spike and the abolition of the fast after-hyperpolarization reflecting the blockage of the Ca^{2+} conductances. *b* and *c*, Oscillatory properties of the same cell before and after Ca^{2+} blockage. In normal medium (*b*) the voltage oscillations are clearly apparent (mean membrane potential, -56 mV). The auto-correlogram (inset) has dominant frequency at 10 Hz. Blockade of Ca^{2+} channels (*c*) did not remove the subthreshold oscillatory event, although its frequency diminished to 8 Hz, as demonstrated by the auto-correlogram to the right of the panel. *d*, Ionic current after leakage subtraction after a single voltage-step from rest (-66 mV) to the oscillation voltage level in control Ringer's solution. Three components are visible: an initial inward current (1), a slow outward current (2) and an outward tail (3). *e*, Inward currents obtained by subtracting the total membrane current in the presence of TTX (1μ M) from those obtained for the same depolarizing steps in control Ringer's solution (holding potential, -65 mV). Note that the inward TTX-sensitive current



shows an early component that slowly inactivates (4) and a persistent component (5). *f*, Slow ionic currents blocked by Cs^+ obtained by subtracting the total membrane currents activated by symmetrical voltage steps from -66 mV in the presence of 2 mM Cs^+ and 1μ M TTX from those obtained for the same voltage steps with only TTX in the Ringer's solution.

II of the EC. Delayed and anomalous rectification was generated by inward and outward current pulses, respectively. The early depolarizing peak, which could fire the cell, was followed by membrane oscillations. The rebound potential after the hyperpolarizing pulse could generate full spikes. Eight of ten neurons injected with biocytin were identified morphologically as ECIIs and demonstrated the electrophysiological properties described here; the other two neurons were morphologically and electrophysiologically different.

The ECIIs neurons analysed in detail (21 of 63) had a mean input resistance of 25.4 ± 9.1 M Ω (mean $\pm$ s.d.) and a mean resting potential of -64.5 ± 3.5 mV. In addition to fast action

potentials, all 63 cells demonstrated rhythmic subthreshold oscillations. Depolarizing current injection produced sustained sinusoidal-like membrane-potential oscillations at 5.1–11.7 Hz (mean, 8.2 Hz; theta frequencies are 4–12 Hz—see ref. 18). The oscillations had a lower threshold than the fast action potential and reached their maximum amplitude near -59 to -54 mV. In the cell of Fig. 1*d* (upper trace) prominent oscillations occurred near -57 mV; the dominant frequency was 8 Hz (inset). Oscillations at three levels of depolarization from rest (-64 mV) are shown in Fig. 1*c*. Superfusion with tetrodotoxin (TTX, 1μ M; $n=26$; lower trace in *d*) blocked the oscillations, as did replacing the extracellular Na^+ with choline ($n=2$), or loading

with the lidocaine derivative QX 314 (15–100 mM; $n = 15$)^{19,20}. These results indicate that the membrane-potential oscillations in ECIIscs are due to the activation of a persistent Na^+ conductance^{10,20–23}. This is in contrast to neurons⁶ in which TTX-insensitive membrane-potential oscillations are completely abolished by blocking the voltage-dependent Ca^{2+} conductances.

The activation of a Na^+ conductance is necessary and sufficient for membrane-potential oscillation as shown in Fig. 2. In *a*, a trace recorded in normal Ringer's solution and one recorded 10 min after changing to a Cd^{2+} (0.3 mM)– Co^{2+} (2 mM) solution are superimposed. Note the increased spike duration and block of the fast after-hyperpolarization in Cd^{2+} – Co^{2+} . Before application of channel blockers, clear membrane-potential oscillations were elicited by depolarization to -56 mV (*b*); 10 min after Cd^{2+} superfusion, synaptically evoked responses were abolished (data not shown) but the subthreshold membrane-voltage oscillation remained (*c*). The frequency of the oscillation was reduced from 10 to 8 Hz, however. Similar results were obtained in all ECIIscs superfused with Cd^{2+} – Co^{2+} ($n = 8$), or with low Ca^{2+} (0 mM Ca^{2+} and 0.5 mM EGTA).

The ionic currents underlying the oscillation of membrane potential were investigated using the single-electrode voltage-clamp technique ($n = 15$). The current flowing during a voltage-clamp step from rest to the oscillation voltage (Fig. 2*d*) generated an initial inward current (1), a slow outward current (2) and an outward tail current (3). The sodium current (I_{Na}) was deduced by subtracting the membrane currents recorded in the presence of TTX (1 μM) from those generated by the same voltage steps in control Ringer's solution (Fig. 2*e*). The I_{Na} showed a fast, slowly inactivating component (4) and a persistent component (5). Both of these components increased with an increase in the size of the voltage-clamp pulses. Persistent²⁰ and slowly inactivating²⁴ inward Na^+ currents have been described in neocortical pyramidal cells.

A slow inward current activated by membrane hyperpolarization, and a slow outward current activated by subthreshold membrane depolarization (Fig. 2*f*) were observed in the presence of TTX. In every case, both currents were blocked by 2 mM Cs^+ ($n = 6$), and thus are probably due to a rectifier such as the I_Q in other cells of the central nervous system^{25,26}. After addition of TTX and Cs^+ , no voltage-dependent currents were observed with voltage-clamp steps of -65 mV to -50 mV (data not shown).

The voltage-clamp data indicates that the activation of an inward Na^+ current and inactivation of a I_Q -like current contribute to the subthreshold membrane-potential oscillations in ECIIscs. Although blocking Ca^{2+} channels decreased the oscillation frequency, Ca^{2+} conductance activation is not necessary to generate oscillations. It is important to note that the final amplitude and shape of the oscillations will be influenced by the membrane time constant, which may vary as the effective membrane resistance changes with activation of the conductances.

The results indicate that a voltage-sensitive subthreshold Na^+ -dependent membrane oscillation underlies the generation of theta rhythm by ECIIscs. The presence of this intrinsic oscillation in ECIIscs is consistent with the *in vivo* discharge pattern of the EC layer-II neurons which is characterized by a mean frequency lower than that of the theta rhythm and a firing that is phase-locked to the theta wave³.

Thus, the generation of an invariant rhythmicity in the brain such as the limbic theta rhythm does not require a common ionic mechanism nor a common anatomical cell type. □

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Reduced Nm23/Awd protein in tumour metastasis and aberrant *Drosophila* development

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TUMOUR metastasis is the principal cause of death for cancer patients¹. We have identified the *nm23* gene, for which RNA levels are reduced in tumour cells of high metastatic potential^{2–5}. In this report we identify the cytoplasmic and nuclear Nm23 protein, and show that it also is differentially expressed in metastatic tumour cells. We also find that the human Nm23 protein has sequence homology over the entire translated region with a recently described developmentally regulated protein in *Drosophila*, encoded by the *abnormal wing discs (awd)*^{6–8} gene. Mutations in *awd* cause abnormal tissue morphology and necrosis and widespread aberrant differentiation in *Drosophila*⁶, analogous to changes in malignant progression. The metastatic state may therefore be determined by the loss of genes such as *nm23/awd* which normally regulate development.

The *nm23* complementary DNA was identified by differential hybridization to messenger RNA from K-1735 melanoma lines of varying metastatic potentials²; *nm23* RNA levels were uniformly reduced in high metastatic potential rodent cell lines (Fig. 1*a*). Human infiltrating ductal breast carcinomas from patients with lymph node metastases and 25% of non-metastatic tumours that were oestrogen receptor negative and poorly differentiated, also contained reduced *nm23* RNA levels⁵.

The murine pnm23-1 cDNA clone² contains an open reading frame that predicts a protein of relative molecular mass 17,180 (17.18K). Affinity-purified anti-Nm23-peptide antibodies were prepared to the proposed N-terminus (peptide 10), internal hydrophilic section (peptide 11) and 5' untranslated region (peptide 65) (Fig. 1*b*). Antibodies to peptides 10 and 11, but not 65, recognized a 17K band from lysates of the low-metastatic-potential K-1735 clone 19 cells on western blots (Fig. 2), in agreement with the predicted Nm23 protein size. Binding of the

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antibodies was specific, as it was inhibited by a molar excess of synthetic peptide (Fig. 2b) and an affinity purified antibody to a non-Nm23 peptide recognized a protein of different molecular weight (Fig. 2a). A 34K band was also specifically inhibited by excess peptide and may be a Nm23 homo- or heterodimer. In agreement with the RNA data, we found that low-metastatic-potential K-1735 clone 19 cells contained more Nm23 protein than high-metastatic-potential K-1735 TK cells (Fig. 2e).

A human pnm23-H1 cDNA clone also predicts a 17.14K Nm23 protein with 94% identity to the murine sequence (Fig. 1b). Antibodies to peptides 10 and 11 identified 17K and 18.5K bands in lysates of human MCF-7 (ref. 9) breast carcinoma cells (Fig. 3a) and T24 bladder carcinoma cells (data not shown). The Nm23 protein was localized to the nucleus and cytoplasm by subcellular fractionation on a sucrose gradient and immunoperoxidase staining (Fig. 3).

Levels of Nm23 protein were determined on human infiltrating ductal breast carcinomas of varying metastatic potentials, for which *nm23* RNA levels were previously quantitated by *in situ* hybridization⁷. Of seventeen tumours examined, an 89% concordance of positive or negative Nm23 protein immunoperoxidase staining with high or low *nm23* RNA content, respectively, was observed. Figure 3c, d shows immunoperoxidase staining on sections of two tumours, one with a low *nm23* RNA content and multiple lymph node metastases, and a non-metastatic carcinoma with high *nm23* RNA content.

The 17K human Nm23 protein exhibited an extraordinary 78% identity to the predicted amino-acid sequence of the recently reported *Drosophila* developmental gene *abnormal wing discs* (*awd*)^{6,8} throughout their entire translated sequences (Fig. 4a). A null mutation of *awd* causes abnormalities in development, leading to death in the prepupal stage⁶. Only the translated

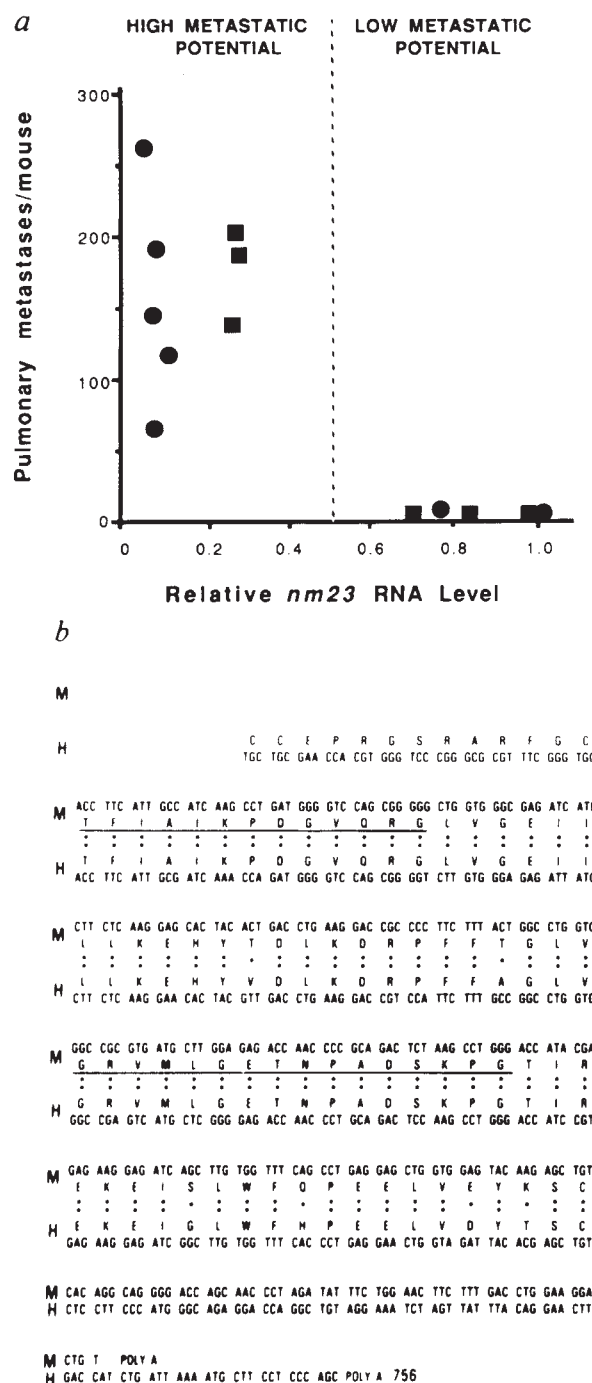


FIG. 1 Complementary DNA sequence and RNA expression of *nm23*. **a**, Comparison of *nm23* RNA levels and tumour metastatic potentials. Levels of *nm23* RNA in K-1735 murine melanoma cell lines (circles) and c-Ha-ras +/- Ad2 E1a transfected rat embryo fibroblast cell lines (squares) were quantitated densitometrically from northern blots^{2,3}. The highest RNA level in each group was arbitrarily set at 1.0. Levels of *nm23* RNA were plotted against the experimental metastatic potential of each cell line, defined as the number of pulmonary metastases resulting from injection of 5×10^4 viable tumour cells into the lateral tail veins of nude mice². **b**, DNA and predicted amino-acid sequences of murine pnm23-1 and human pnm23-H1 cDNA clones used for the production and purification of antibodies. In the murine sequence the three peptides synthesized and used as antigens for anti-Nm23 antibody production are underlined: peptide 10 from the N-terminus (bases 46-99), peptide 11 from an internal hydrophilic section (bases 297-348) and peptide 65 from the predicted 5' nontranslated region (bases 1-42). Predicted methionines are in bold and Kozak's optimal translation initiation sequence¹² is boxed. To identify the human cDNA clone, a *Hpa*II restriction fragment of pnm23-1 was used to screen a human fibroblast Okayama-Berg library. Dideoxy-chain termination sequencing was used to determine the sequence of the pnm23-H1 insert after insertion into M13 phage.

FIG. 2 Western blot identification of the murine Nm23 protein in K-1735 murine melanoma clone 19 cells. *a*, Demonstration of a 17K Nm23 protein in a clone 19 lysate using anti-peptide 11 antibody, but not with a control antibody. The specificity of anti-peptide 11 antibody was demonstrated by its reactivity to 0.1 μg of a BSA-peptide 11 glutaraldehyde-linked conjugate, but not to glutaraldehyde-fixed BSA. *b*, Inhibition of anti-peptide 11 antibody binding to a clone 19 lysate by a 400 $\times$ molar excess of peptide 11, but not by an unrelated peptide. *c*, Demonstration of a 17K Nm23 band in a clone 19 lysate using anti-peptide 10 antibody. *d*, Confirmation of methionine (base 43) as the translation initiation point. A western blot containing a clone 19 lysate was incubated with anti-peptide 65 antibody, directed towards the putative 5' untranslated region of *nm23*. As a positive control, a duplicate section of the same nitrocellulose blot was incubated with an equivalent concentration of anti-peptide 11 antibody. *e*, Low-metastatic-potential K-1735 melanoma clone 19 cells contain greater Nm23 protein levels than related, high-metastatic-potential K-1735 melanoma TK cells. Three Nm23 peptides were synthesized on a Bioscience 9600 peptide synthesizer using standard Merrifield solid phase t-boc procedures. Rabbit antisera to peptide-BSA glutaraldehyde-linked complexes were prepared and affinity-purified as described¹³. Cells were lysed in 0.5% (v/v) NP40 detergent 1.0 μg ml^{-1} aprotinin in 0.9% (w/v) NaCl, 20 mM Tris, pH 7.5 (TBS). 30 μg of total cellular protein was electrophoresed in a 14% SDS-polyacrylamide gel, together with 0.1 μg of BSA-peptide glutaraldehyde-linked conjugates as indicated and prestained markers, transferred to nitrocellulose and a western blot developed with anti-Nm23 antibody and a peroxidase-conjugated goat anti-rabbit IgG. The sequence of the control peptide is DKPMGPLLVATFWPEIPEK (single-letter amino-acid code), and the control antibody was directed to a synthetic peptide of the sequence AIVAIENPADVS-VISSRNTG. Quantitation of the tumour metastatic potentials of K-1735 melanoma clone 19 and TK lines has been reported².

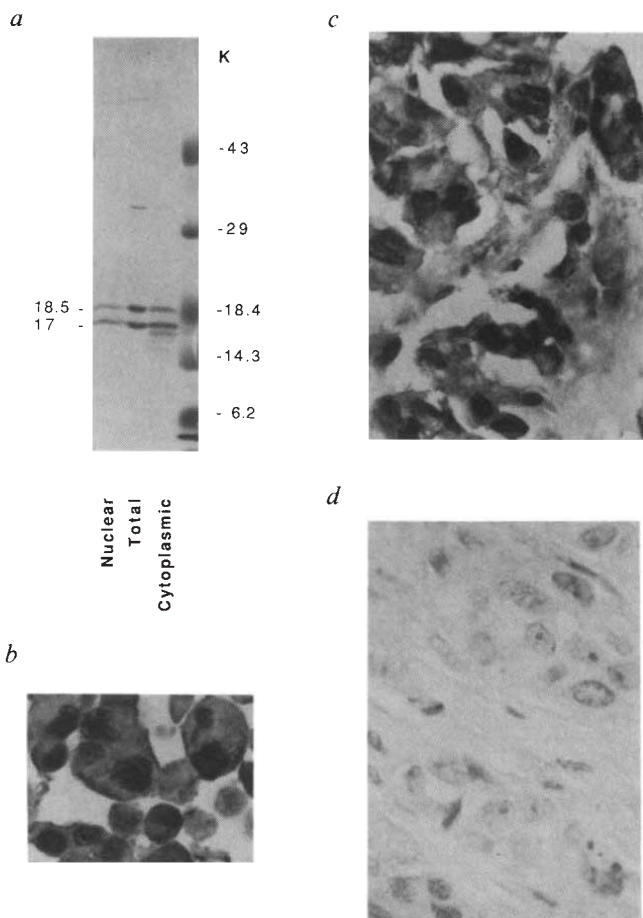
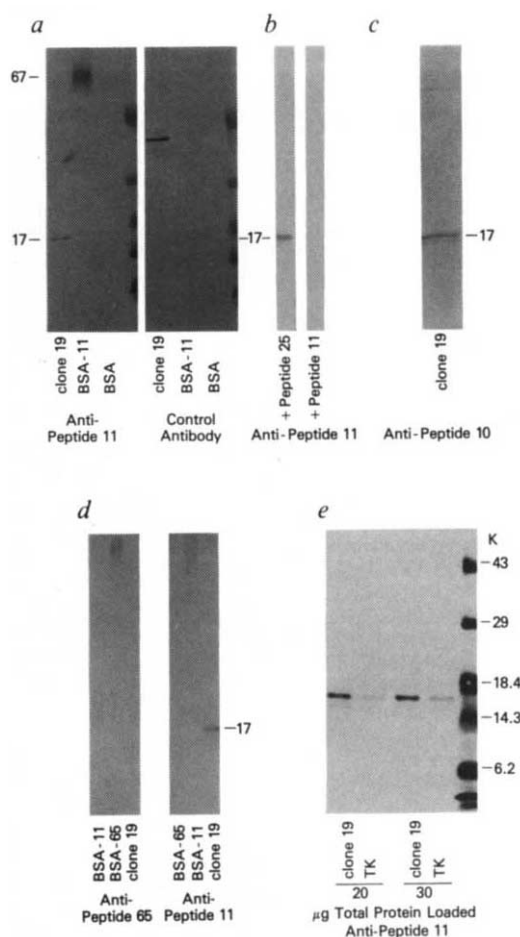
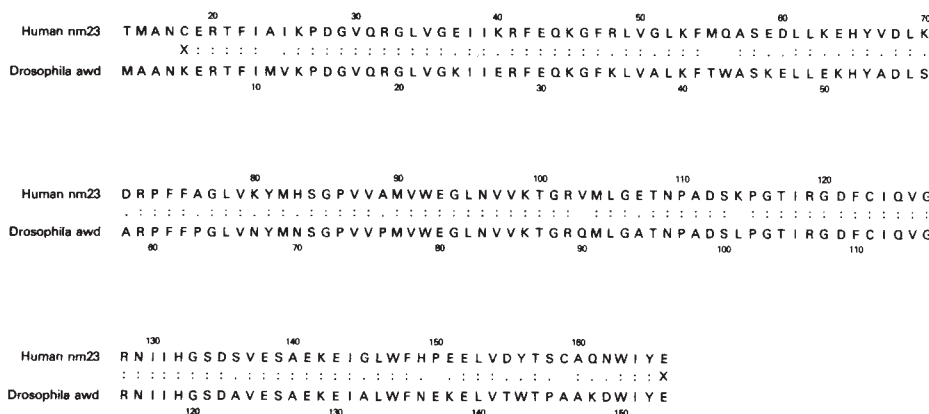


FIG. 3 *a*, Nuclear and cytoplasmic localization of human Nm23 protein. A western blot containing lysates of human MCF-7 breast carcinoma cells, and nuclear and cytoplasmic fractions of these cells, was incubated with anti-peptide 11 antibody. *b*, Immunoperoxidase staining of murine K-1735 clone 19 cells using anti-peptide 11 antibody. *c* and *d*, Reduced Nm23 protein levels in high-metastatic-potential human infiltrating ductal breast carcinomas. Formalin-fixed paraffin embedded sections of seventeen breast carcinomas of varying metastatic potentials, for which *nm23* RNA levels were previously quantitated by *in situ* hybridization⁵, were stained with anti-peptide 11 antibody to determine Nm23 protein content. Protein levels were scored on a 0–3 scale by pathologists, blind to histopathological and *nm23* RNA data; sections with a 2–3 rating were judged to be positive. *c*, Non-metastatic breast carcinoma with high *nm23* RNA level. *d*, Breast carcinoma with multiple lymph node metastases and low *nm23* RNA level. MCF-7 cells were separated into nuclear and cytoplasmic fractions on a discontinuous sucrose gradient¹⁴. Electron microscopic evaluation of a portion of the nuclear pellet failed to reveal any cytoplasmic organelles. The cytoplasmic fraction was dialysed to TBS, pH 7.5, and concentrated. Total protein (30 μg) in NP40 lysates of whole MCF-7 cells, nuclear fraction and cytoplasmic fraction were electrophoresed in a 14% SDS-polyacrylamide gel, and a western blot developed using anti-peptide 11 antibody.

a



b

17 K



FIG. 4 Comparison of Nm23 and Awd protein sequences. *a*, The predicted amino-acid sequences of the human Nm23 (Fig. 1) and *Drosophila* abnormal wing discs (Awd)⁵ proteins were compared using the BIONET program and the SWISS-PROT database (release 10.0) by the method of Pearson and Lipman¹⁵. 78% (115/148) of the predicted amino acids analysed were identical between Awd and Nm23. Of the remaining 33 non-identical amino acids, 10 are conserved in charge, and 16 have a similar hydrophobicity profile, bringing the total similarity of the Awd and Nm23 proteins to 95%.

b, Demonstration of anti-Nm23 peptide 11 antibody recognition of Awd protein in a *Drosophila* embryo lysate. *Drosophila* embryos were homogenized in sample buffer (20% glycerol, 3% SDS, 3% β -mercaptoethanol, 5 mM Tris HCl, pH 7.4, and boiled for 10 min). Proteins were separated on a 12.5% SDS-polyacrylamide gel, and transferred to a nitrocellulose filter. The filter was incubated with anti-peptide 11 antibody followed by ¹²⁵I-labelled protein A. Bands were visualized by autoradiography.

protein sequences of *awd* and *nm23* are conserved, as the predicted amino acids flanking the translated sequences are only 12% identical at the 5' end, and 4% identical at the 3' end. In agreement with the homology of predicted *nm23* and *awd* translated amino-acid sequences, antibody to Nm23 peptide 11 recognized a 17K protein in lysates of *Drosophila* embryos (Fig. 4b).

The reduced level of Awd protein in *awd* mutant *Drosophila* larvae⁸ paralleled decreases of *nm23* in cancer metastasis. The mutant *awd* phenotype includes: (1) abnormal morphology of the wing discs, larval brain, and proventriculus; (2) aberrant differentiation of the wing, leg, and eye-antenna imaginal discs and ovaries; (3) cell necrosis, predominantly in the wing discs; and (4) heterogeneity in the pattern of abnormal morphology and cell death, to the extent that two imaginal discs from the same larva can be different⁶. Similar phenotypic alterations are also observed in tumour progression^{10,11}. Transformation of the mutant *Drosophila* germ line with wild-type *awd* DNA resulted in normal morphology and development⁷. The data indicate that the numerous phenotypic changes in tumour metastasis may result from alterations in the expression of relatively few genes.

The remarkable degree of conservation between Nm23 and Awd proteins implies that they are functionally homologous. The *nm23/awd* gene may contribute to the normal development of cells. Loss of *nm23/awd* expression may lead to a disordered state, favouring aberrant development or cancer progression. Decreased levels of *nm23/awd* in cancer metastasis and developmental mutants may provide a molecular link between these two processes. □

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Recognition of pre-processed endogenous antigen by class I but not class II MHC-restricted T cells

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CLASS I and class II MHC-restricted T lymphocytes recognize non-native forms of antigen^{1–7}. The presentation of antigen to these two classes of T lymphocytes can occur through distinct pathways^{8–10}. Several mechanisms, including differences in antigen processing in different intracellular compartments, have been proposed to account for these pathway differences^{9–14}. Here we describe a T-cell epitope located on the influenza virus haemagglutinin, which is recognized by both class I and class II MHC-restricted cytolytic T lymphocytes (CTL). When expressed *de novo* in target cells, from a synthetic minigene¹⁵ encoding only the epitope, this pre-processed antigenic site is recognized by class I but not class II MHC-restricted T lymphocytes, even though target cells treated with the exogenously introduced peptide can be recognized by both classes of T cells. Because endogenous expression of the pre-processed antigenic fragment results in differential presentation to class I and class II MHC-restricted CTL, differences between the two different pathways of presentation could lie

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not at the level of processing but at the level of targeting and/or interaction of processed antigen with MHC.

We previously reported that the region between A/JAPAN/305/57 haemagglutinin (HA) amino-acid residues 227 and 394 probably contains an epitope(s) recognized by H-2^k restricted CTL (ref. 16). We have now identified an HA site recognized by H-2K^k-restricted CTL which can be mimicked by a synthetic peptide of HA residues 252-271 (HA252-271). Unexpectedly, this site was also recognized by CD4⁺ HA-specific CTL^{8,17} restricted by I-A^d. Figure 1 shows specific recognition of target cells treated with HA252-271 peptide by both an HA-specific H-2K^k-restricted CTL line (CBA) and three I-A^d-restricted cytolytic clones (clones N2.7, U5 and V4).

Analyses using a series of truncated peptides spanning HA252-271 are summarized in Table 1. The smallest peptides optimally recognized by the class I-restricted CTL and class II-restricted CTL were those of HA residues 255-271 (HA255-271) and HA residues 257-271 (HA257-271), respectively.

The pathways for antigen presentation to class I and class II-restricted T lymphocytes seem to be distinct⁸⁻¹⁰ in that

exogenous antigen, taken up and processed presumably in an endosome, was efficiently recognized by class II-restricted T cells whereas exogenous antigen introduced into the cytosol or endogenous antigen expressed *de novo* and processed in the cell was efficiently recognized by class I-restricted T cells. Because the HA252-271 peptide was recognized by both T-cell subsets, we investigated whether exogenous HA and endogenous HA could be presented to both class I and class II-restricted, HA252-271-specific CTL. Both class I and class II-restricted T cells recognized influenza infected target cells (Table 2). Cells treated with non-infectious influenza virus (UV-JAP) as a source of exogenous HA were recognized by class II-restricted T cells only, whereas cells infected with a recombinant vaccinia vector expressing the JAP gene for HA (VV-HA)^{18,19} as a source of endogenous HA, were recognized only by class I-restricted T cells. Vaccinia virus infection of target cells did not abolish processing and presentation of HA to class II-restricted T cells by the endosomal pathway during the 6-h cytotoxicity assay (ref. 8, and unpublished observations).

Because target cells treated with exogenously added HA252-271 peptide (Fig. 1) were able to be recognized by both class I and class II-restricted T cells, we determined whether an endogenously expressed HA252-271 site could bypass any block in antigen processing and be recognized by class I and class II-restricted T cells. A minigene (MD252-271) encoding a translation start Met-Asp followed by the 20 residues of the HA252-271 peptide site was constructed¹⁵ and inserted into a vaccinia virus expression vector (Fig. 2). Like target cells expressing mutant HA lacking the signal sequence²⁰, targets expressing the MD252-271 minigene were recognized by class I-restricted T cells. They were not recognized, however, by class II-restricted T cells (Table 2). The synthetic peptide HA MD252-271 (MDTINFESTGNLIAPEYGFKIS, Fig. 2) was recognized with the same efficiency as the HA252-271 peptide, and the class II-restricted T cells recognized both peptides with a higher efficiency than class I-restricted T cells (Fig. 1).

Our results demonstrate that differences in antigen presentation to class I and class II-restricted T cells are probably not due to processing differences, but rather either reflect differences

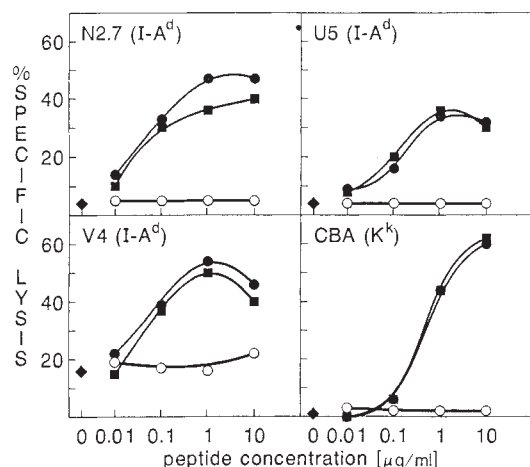


FIG. 1 Recognition of peptide-treated LK35.1 target cells by class I and class II MHC-restricted HA-specific T lymphocytes. Three CD4⁺ I-A^d-restricted CTL clones (U5, V4 and N2.7)^{8,17} and a CD8⁺ H-2K^k-restricted long term CTL line (CBA) were tested for lytic activity in a standard ⁵¹Cr release assay on class I and class II MHC positive H-2K^d hybrid LK35.1 cells untreated (◆) or incubated with synthetic peptides HA252-271 (■), HA260-271 (○), or HA MD252-271 (●). See Fig. 2 for peptide sequences. METHODS. The I-A^d-restricted clones U5, V4 and N2.7 were derived from infectious A/JAP/57 virus-immune BALB/c splenic precursors by limiting dilution and subsequent expansion of clonal progeny in IL-2 supplemented medium^{8,17}. Clones were maintained by subculture in the presence of A/JAP/57 virus-infected, irradiated (2,000 rad) BALB/c splenocytes without exogenous growth factor¹⁷. The long-term H-2K^k-restricted CTL line (CBA) was established from CBA/J mice immunized with an A/JAP/57 HA-expressing recombinant vaccinia virus¹⁸ and subsequent *in vitro* stimulation of immune splenocytes with syngeneic irradiated splenocytes infected with A/JAP/57 virus. The bulk culture was re-stimulated bi-weekly with irradiated A/JAP/57-infected CBA/J splenocytes in the presence of exogenous growth factor. MHC restriction of the T-cell line and clones was determined by *in vitro* cytotoxicity assays using target cells expressing transfected class II MHC genes¹⁷ and by antigen-driven clonal proliferation using infected splenocytes stimulators from recombinant inbred strains¹⁷. Cytotoxicity assays were performed for 4-6 h at 37 °C at three effector to target ratios (results for the ratio 5:1 only are shown) at the indicated concentration of purified synthetic peptide. Per cent specific lysis of target cells was calculated as described^{8,17}. Peptides were synthesized (Applied Biosystems Peptide synthesizer) and when necessary, further purified by reverse phase HPLC on a C-18 column. Sequences were verified by amino-acid composition analysis and direct peptide sequencing. In control experiments (not shown) class I and class II-restricted clones and lines did not recognize adjacent partially overlapping HA240-259 and HA265-284 synthetic peptides as well as more than 10 peptides corresponding to unrelated portions of the A/JAP/HA.

TABLE 1 Recognition of a nested series of peptides by the HA252-271-reactive H-2K^k and I-A^d-restricted T cells.

Synthetic peptide	MHC class I (K ^k restricted)	MHC class II (I-A ^d restricted)
252 255 260 265 271 TINFESTGNLIAPEYGFKIS	+	+++
MDTINFESTGNLIAPEYGFKIS	+	+++
NLIAPEYGFKIS	-	-
STGNLIAPEYGFKIS	-	+++
FESTGNLIAPEYGFKIS	+++	+++
TGNLIAPEYGFK	-	++
ESTGNLIAPEY	-	++
NFESTGNLIAPE	++	-
TINFESTGNLIAPEYGF	++	++
TINFESTGNLIA	+	

Results are a summary of analyses of the recognition of these peptides by the H-2K^k-restricted CTL line (CBA) and three clones derived from this line and by the I-A^d-restricted T-cell clones V4 and N2.7. Assays were carried out at three effector to target ratios for 6 h on LK35.1 target cells treated with the indicated peptides at concentrations from 0.01 µg ml⁻¹ to 10 µg ml⁻¹. Single letter code is used to designate specific amino acids. Symbols reflecting the peptide dose range at which target cell lysis was one-half maximal: -, No specific lysis of peptide-treated targets up to concentrations of 10 µg ml⁻¹; +, Half-maximal lysis at concentrations between 1 µg ml⁻¹ to 10 µg ml⁻¹; ++, Half-maximal lysis between 0.1 µg ml⁻¹ to 1 µg ml⁻¹; +++, Half-maximal lysis between 0.01 µg ml⁻¹ to 0.1 µg ml⁻¹.

TABLE 2 Haemagglutinin presentation to class I and class II MHC-restricted T cells

Effectors	Uninfected	HA255-271	% Lysis of I-A ^d /H-2K ^k LK35.1 target cells Infectious A/JAP/57	UV-JAP	VV(SC11)	VV(HA)	VV(MD252-271)
Class I-restricted							
CBA line	0	71	67	3	1	45	48
K8-1	4	68	63	5	3	34	41
K8-4	0	66	64	1	1	41	49
K8-5	0	68	71	1	1	41	50
Class II-restricted							
V4	7	66	71	59	6	12	3
U5	9	49	60	46	5	4	2
G1	3	5	78	66	1	11	1

LK35.1 cells were labelled with ^{51}Cr and served as target cells in a 6-h cytolytic assay (see Fig. 1 legend). The class I-restricted effector cells were the H-2K^k-restricted HA252-271-reactive CTL line and three CD8⁺ clones derived from the line (K8-1, K8-4, K8-5). The class II-restricted effectors were the I-A^d-restricted HA252-271-reactive clones V4 and U5 and a CD4⁺ HA-specific cytolytic clone of BALB/c strain origin G1^{8,17}, which is directed to an unrelated site on the A/JAP/57 HA (L.A.M., unpublished result). Assays were performed at effector to target ratios of 1:1 and 10:1 (results for 10:1 ratios are shown). Values are the mean % lysis of triplicate determinations. Standard errors were <5% of mean values in all cases and are omitted. The HA255-271 target cell group consists of uninfected LK35.1 target cells maintained in the presence of the peptide HA255-271 at a concentration of $1\text{ }\mu\text{g ml}^{-1}$ throughout the assay. The A/JAP/57 infectious target cells were infected with A/JAP/57 virus at a multiplicity of infection (MOI) of 10. The UV-JAP target cell group was treated with UV-inactivated A/JAP/57 virus⁸ at a concentration of 1,000 HAU (haemagglutinating units) per 10^6 target cells. The VV(SC11) target cell group was infected with a recombinant vaccinia virus containing only the plasmid pSC11 insertion vector²⁷. The VV(HA) and VV(MD252-271) target cell groups were infected with recombinant vaccinia containing the full-length influenza A/JAP/57 HA gene or the MD252-271 minigene (Fig. 2) at an MOI of 50.

in the capacity of class I and class II MHC molecules in an intracellular compartment to bind antigenic moieties, or reflect the absence of a given MHC class in the compartment containing processed antigen. For processing and interaction with class II MHC, antigen expressed *de novo*, similarly to nascent HA, may need to be targeted into an endosomal compartment. The failure of newly synthesized influenza HA, which is exported to the cell surface by the secretory pathway^{21,22}, to be recognized by class II-restricted T cells presumably reflects the long half-life and slow recycling of cell-surface A/JAP/57 HA²³ into endosomes/lysosomes.

The product of the MD252-271 minigene similarly to the full length endogenous HA, seems to sensitize cells exclusively for

class I-restricted T cell recognition. An attractive hypothesis to account for these results is that the MD252-271 minigene product is directed from the cytoplasm into a translocation competent compartment (possibly the endoplasmic reticulum^{24,25}) by a transporter mechanism³, where it can then bind to nascent class I MHC molecules. Accordingly, class II MHC in the compartment must not be functionally competent to be charged by this product (for example, class II MHC is blocked by invariant chains²⁶), or rapidly egresses from this compartment. Furthermore, the minigene product must not have access to class II MHC on the cell surface or in an endosomal compartment where the class II MHC presumably interacts with antigen fragments. □

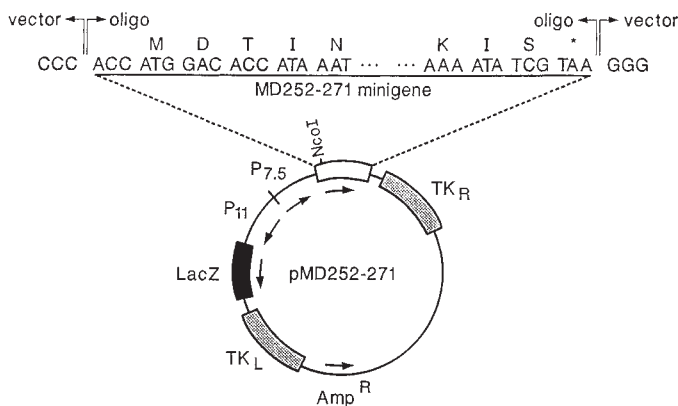


FIG. 2 Construction of the MD252-271 vaccinia virus expression vector. METHODS: Two complementary 72-base pair (bp) oligonucleotides ($2.5\text{ }\mu\text{g}$) encoding a translation start site (M) plus an aspartic acid (D) joined to the 20-amino-acid sequence represented by HA252-271 were annealed together in 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT by heating at $100\text{ }^{\circ}\text{C}$ for 3 min and slowly cooling to $25\text{ }^{\circ}\text{C}$. The annealed oligonucleotides were ligated into the *Sma*I site of the vaccinia virus expression vector plasmid pSC11¹⁷. Colonies were screened for presence of the insert in the proper orientation to the vaccinia early-late P_{7.5} promoter by restriction-enzyme digests and sequencing of double-stranded DNA with Sequenase (United States Biochemical). The MD252-271 minigene construct was introduced into the vaccinia virus thymidine kinase (*tk*) locus by using standard techniques^{15,27}.

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How some T cells escape tolerance induction

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A FEATURE common to many animal models of autoimmune disease, for example, experimental allergic encephalomyelitis¹, experimental autoimmune myasthenia gravis² and collagen-induced arthritis³, is the presence of self-reactive T cells in healthy animals, which are activated to produce disease by immunization with exogenous antigen. It is unclear why these T cells are not deleted during ontogeny in the thymus and, having escaped tolerance induction, why they are not spontaneously activated by self-antigen. To investigate these questions, we have examined an experimental model in which mice are tolerant to an antigen despite the presence of antigen-reactive T cells⁴. We find that the T cells that escape tolerance induction are specific for minor determinants on the antigen. We propose that these T cells evade tolerance induction because some minor determinants are only available in relatively low amounts after *in vivo* processing of the whole antigen. For the same reason, these T cells are not normally activated but can be stimulated under special circumstances to circumvent tolerance.

T lymphocytes are stimulated by denatured antigen bound to cell-surface glycoproteins encoded by the major histocompatibility complex (MHC)⁵. Antigens are internalized by antigen-presenting cells (APC) and partially degraded to produce individual determinants which are recycled to the cell surface. Synthetic peptides have been used to define several determinants on hen eggwhite lysozyme (HEL) that induce T-cell responses in B10.A mice. In addition to the finding that some peptides were non-immunogenic, it was found that the contribution of individual peptide determinants to the HEL-induced response was unequal, and that some peptides recalled greater responses *in vitro* than others⁶⁻⁸. Thus, T cell-inducing determinants can be divided into major and minor categories. The *in vitro* proliferative response to HEL and to several peptides following HEL immunization is shown in Fig. 1. Peptides 20-35 and 46-61 contain major determinants, whereas peptides 1-18 and 30-53 recall only weak responses, indicating that they bear minor determinants.

Intravenous injection of 2 mg of HEL in saline induces tolerance in adult B10.A mice. Although these tolerant mice do not respond to immunization with HEL in complete Freund's adjuvant, significant proliferative responses can be stimulated by immunization with the cyanogen bromide fragments corresponding to HEL amino-acid residues 13-105 and 106-129 (ref. 4). The response generated by fragment immunization can be recalled *in vitro* by whole HEL, as well as by the fragment itself. Thus, HEL-reactive T cells are present in these tolerant mice, but are not activated by *in vivo* challenge with HEL.

Adult tolerance to HEL was examined further by immunizing HEL-tolerant mice and normal control mice with the HEL peptides 1-17, 30-53, 20-35, 46-61, 74-96 and 81-96, and measuring the responses recalled *in vitro* by the peptide immunogen and by HEL (Fig. 2). The normal mice responded well to each peptide, but the tolerant mice responded only to peptides 30-53, 74-96 and 81-96, which bear minor determinants. The T cells specific for the 1-17, 20-35 and 46-61 peptides seem to have been rendered tolerant. The lack of response to the 20-35 and 46-61 peptides is important because they contain major determinants (Fig. 1) and the loss of reactivity to them affects the overall level of response to HEL. Conversely, the preservation of responsiveness to peptide 30-53 in tolerant animals does not affect the lack of response to whole HEL, because in fact there is very little proliferation by HEL-

primed lymph node cells to peptide 30-53 *in vitro*. Tolerant mice also respond to the 74-96 peptide, which contains two minor determinants, although the response is lower than in controls. Peptide 81-96, which has one of the two determinants of peptide 74-96, induces a normal response in HEL-tolerant mice. Like peptide 30-53, however, 74-96 and 81-96 make little or no contribution to the normal HEL-induced response^{6,8}.

How do (30-53)- and (74-96)-specific T cells escape tolerance induction? Although it is not currently possible to measure the concentration of antigenic determinants on the surface of APC, there is abundant evidence that suggests that the most important factor dictating the level of response to each determinant is the amount of it bound to MHC-encoded molecules on the cell surface⁶. A major determinant is dominant owing to its efficient processing and capacity to bind an MHC molecule with high affinity. Conversely, a minor determinant may not meet these criteria and only be present in a biologically active form at relatively low levels after immunization with the whole antigen. Responses to these minor determinants can be stimulated by direct peptide immunization, which bypasses the need for anti-

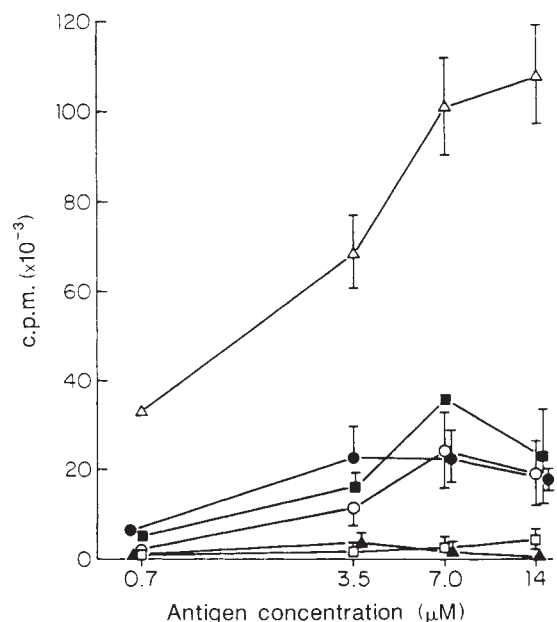


FIG. 1 The proliferative response of HEL-primed lymph node cells (LNC) to HEL and HEL peptides. The LNC from four B10.A mice were pooled and tested for *in vitro* reactivity against HEL (Δ) and the HEL peptides 1-18 (□), 13-35 (●), 20-35 (○), 30-53 (▲) and 46-61 (■) at the concentrations shown. The data are the arithmetic mean of triplicate wells, less the medium-only background. The standard deviation is shown where it is >10% of the c.p.m.

METHODS. B10.A/SgSnJ mice (8 to 14 weeks old) were immunized with 3.5 nmol of HEL or HEL peptide in saline, in a 1:1 emulsion with complete Freund's adjuvant (CFA) in the hind footpads. After 10 days, the popliteal and inguinal lymph nodes were removed and cell suspensions prepared. The LNC were cultured in 96-well plates at 4×10^5 cells per well in HL-1 medium (Ventrex) with the indicated concentrations of antigen. Proliferation was measured by addition of 1 μCi of [³H]thymidine for the last 18 hours of a 5-day culture, and the incorporation assayed by liquid scintillation counting. HEL peptides were synthesized (J. Young, UCLA; M. McMillan and L. Williams, USC) using a model 430A peptide synthesizer (Applied Biosystems). The methodology has been described²³. The peptides were passed over a G10-Sephadex column (Pharmacia) and the peptide fraction further purified as a single peak by HPLC on a preparative C₈ column with a solvent system of 0.1% trifluoroacetic acid and an increasing gradient of acetonitrile. Peptide identity was confirmed by amino-acid composition analysis. The 74-96 peptide preparation gave a single major peak on analytical HPLC and, because of its poor solubility, was not purified further. Peptide 1-17 was a gift from D. Nitecki, Cetus.

gen processing. We propose that the T cells responsive to peptides 30-53 and 74-96 escape tolerance induction because these determinants are poorly presented and do not reach a tolerogenic level. This can be tested by using a greater amount of HEL to induce tolerance, because this should raise the level of each minor determinant to the tolerogenic threshold. The data in Fig. 3 show that high-dose (20 mg i.v.) HEL-tolerant mice mounted a weaker response to peptides 30-53, 74-96 and 81-96 compared with mice tolerized with 2 mg.

What are the mechanisms responsible for HEL tolerance induction in adult mice? The correlation between the level of responsiveness to individual determinants after HEL priming and the ease with which non-responsiveness to them is produced, favours a mechanism of direct clonal inactivation⁹⁻¹³. The ease of tolerance induction to peptide 1-17, a minor determinant, does not seem to fit this mechanism. Peptide 1-17, however, is a major determinant in the H-2^k strain B10.BR (unpublished data). Also, peptide 1-18, but not peptides 30-53 or 74-96, is a dominant determinant in the H-2^k strain C3H, and binds I-E^k with relatively high affinity⁸. Peptides 1-17 and 1-18 have identical activity in B10.A mice (unpublished data). By analogy, it seems likely that peptide 1-17 is presented well in B10.A mice, and that the weak response to it after HEL priming reflects an additional factor influencing determinant hierarchy. This may be a lower frequency of (1-17)-specific T cells in B10.A as compared with B10.BR mice. This in turn could be due to the genetic polymorphisms between these strains in the MHC class I D-region or possibly in the T-cell receptor α -chain region¹⁴. Alternatively, a T suppressor cell-inducing determinant co-existing with a proliferation-inducing determinant on peptide 1-17 could account for unresponsiveness to this peptide¹⁵.

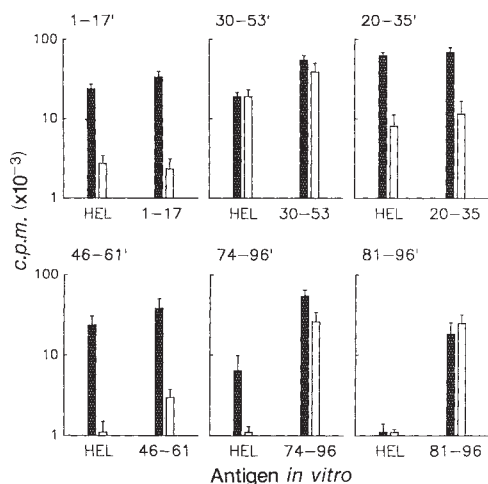


FIG. 2 The response of normal and 2 mg-dose HEL-tolerized B10.A mice to challenge with HEL peptides 1-17, 30-53, 20-35, 46-61, 74-96 and 81-96. Adult B10.A mice (8 to 14 weeks old) were tolerized by intravenous injection of 2 mg HEL in saline. Twelve to 14 days later, control and tolerant animals were immunized with peptide/CFA as described in the legend for Fig. 1. The draining lymph nodes were removed after 10 days and *in vitro* reactivities to HEL and peptide at a concentration of 7 μ M were tested. The culture conditions were identical to those described in Fig. 1. The data shown are the arithmetic means of the individual responses of each mouse in a group to HEL and peptide *in vitro*, less the medium-only background, ± 1 s.e.m. Each group contained 6 to 8 mice, except for the groups immunized with peptide 20-35, which each contained 4 mice. The peptide used for immunization is indicated above each panel. The shaded histograms correspond to the control mice and the open histograms to the HEL-tolerant animals. The average medium-only backgrounds ($\times 10^{-3} \pm 1$ s.e.m.) for control and tolerant mice respectively, are: Peptide 1-17, 4.4 ± 0.9 and 3.1 ± 0.8 ; peptide 30-53, 6.6 ± 0.9 and 5.6 ± 0.7 ; peptide 20-35, 6.1 ± 0.9 and 5.9 ± 1.3 ; peptide 46-61, 3.1 ± 0.4 and 4.8 ± 1.2 ; peptide 74-96, 3.5 ± 1.5 and 1.3 ± 0.3 ; peptide 81-96, 0.3 ± 0.1 and 0.2 ± 0 .

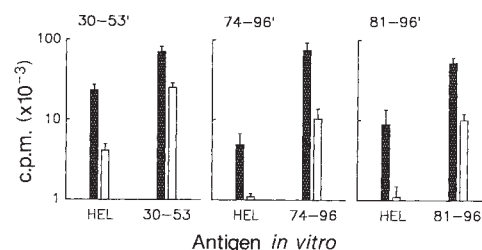


FIG. 3 The response of normal and 20 mg-dose HEL-tolerized B10.A mice to challenge with HEL peptides. Adult B10.A mice were tolerized by intravenous injection of 20 mg HEL. Twelve to 14 days later, control and tolerant animals were immunized with peptide/CFA as described in the legend to Fig. 1. After 10 days, the draining lymph nodes were removed and reactivity to HEL and the appropriate peptide at 7 μ M was tested. The data shown are the arithmetic means of the individual responses of each mouse in the group to HEL and peptide *in vitro*, minus the medium-only background, ± 1 s.e.m. Each group contained 6 to 8 mice. The peptide used for immunization is indicated above each panel. The shaded histograms correspond to the control mice and the open histograms to the tolerant groups. The average medium-only backgrounds ($\times 10^{-3} \pm 1$ s.e.m.) for control and tolerant mice respectively, are: peptide 30-53, 2.3 ± 0.4 and 2.5 ± 0.3 ; peptide 74-96, 1.2 ± 0.3 and 0.9 ± 0.2 ; peptide 81-96, 1.7 ± 0.3 and 1.5 ± 0.2 .

Adult-induced tolerance to HEL in B10.A mice is analogous to several models of autoimmune disease in which an animal not responding spontaneously to self-antigen can be shown to possess self-reactive T cells. Tolerance induction in the thymus requires the presentation of self-antigens to developing T cells (reviewed in ref. 16). The efficiency of presentation, therefore, will be a factor in deciding which T cells are deleted, and so it is probable that T cells specific for some minor self-determinants will evade deletion. These are other mechanisms by which T cells could avoid tolerance induction. Some autoreactive T cells may escape deletion because they express low-affinity receptors. Alternatively, induction of tolerance in the thymus may be incomplete and some autoreactive T cells may mature and migrate to the periphery, where autoreactivity would be prevented by peripheral mechanisms, for example by T cell suppression. Neither of these hypotheses can fully explain our data. There is no evidence suggesting that T cells recognizing minor determinants are of lower affinity than those for major determinants. (In fact, they may be of higher affinity as they are up against lower concentrations of presented peptide.) Also, it is difficult to see how the random escape from deletion by a certain fraction of the T-cell population would lead to the radical changes observed in the specificity of the T-cell response.

In our model, tolerance to many self-proteins, except those at high concentrations, will not be complete, but will be restricted to the major T cell-inducing determinants. A similar model based on theoretical considerations has been proposed elsewhere¹⁷. We propose that the pathogenic determinants on self-antigens are not typical major T cell-inducing determinants, but will resemble minor ones. Responses to minor determinants are not stimulated under normal conditions, but such responses may be induced by a cross-reactive major determinant on a bacterial or viral protein¹⁸, by a super-antigen¹⁹ or mitogen, or by self-protein in an altered form²⁰. Once these responses have been initiated, they may be restimulated by self-antigen because the conditions required to recall memory T-cell responses seem to be less stringent than those required to activate naïve cells²¹. This can be seen in the HEL system: immunization of B10.A mice with peptides 30-53 or 74-96 primes for helper T cells that are recruited to help native HEL-specific B cells upon challenge with HEL *in vivo*²². Responses to poorly presented minor determinants can therefore be important *in vivo* and the stimulation of such responses in tolerant mice will circumvent T-cell unresponsiveness to the whole antigen. □

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Complex lymphoid and epithelial thymic tumours in *Thy1-myc* transgenic mice

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T-LYMPHOCYTE development takes place mainly in the thymus, where stromal cells of epithelial and haemopoietic origin are involved in inductive and selective mechanisms, which enable specific lymphocyte populations to migrate to the periphery and establish a network of immune responses. Experiments with intact animals have clarified the precursor–product relationships between thymocyte subpopulations, but the molecular mechanisms of cell interactions in the thymus are difficult to study *in vivo*. In an attempt to expand thymic cell populations *in vivo* and maintain them *in vitro* for such studies, we directed high levels of expression of the murine *c-myc* proto-oncogene in transgenic mice by inserting it into the mouse *Thy-1* transcriptional unit. Such mice develop thymic tumours which contain proliferating thymocytes and, interestingly, expanded populations of epithelial cells. Both cell types can be maintained *in vitro*.

Thy-1 is a surface glycoprotein expressed in a variety of mouse tissues, notably thymus and brain¹, whereas the product of the *c-myc* proto-oncogene is a nuclear protein which has been associated with malignancies in a variety of tissues². Deregulation of *myc* expression can lead to cell proliferation and immortalization/transformation^{3–8} and has been used for experimental tissue directed oncogenesis^{9–15}. In some cases cells from such tumours were able to grow *in vitro*^{11,16}.

The expression of *c-myc* was directed to the thymus¹⁷ by placing exons 2 and 3 of the mouse *c-myc* gene into the first intron of the mouse *Thy-1* gene (Fig. 1a). This transcription

unit does not have the *myc* attenuation sequences^{18,19} and results after splicing in a hybrid messenger RNA containing coding sequences for the complete *c-myc* gene product only. Fertilized mouse eggs were injected and 13 transgenic mice were obtained, containing 1–20 copies of the transgene. Three of the animals (TM1, TM28 and TM29) died in the first week after birth before detailed analysis was carried out. The other mice, except TM7 and TM6L (a low copy (1–2) number offspring of TM6), developed breathing difficulties at the age of 8 to 12 weeks, caused by large thymic tumours and three had metastasis in peripheral lymphoid organs. Two of six tumours from founder mice tested caused subcutaneous tumours in histocompatible animals. Three of eight tumours from founder mice tested were established as long-term (>6 months) cell cultures. Two of the founder mice (TM6 and TM25) were bred extensively and all of the descendants developed large thymic tumours by 9–14 weeks.

To determine whether the normal *Thy-1* transgene expression pattern, in brain and thymus, is observed for the hybrid gene, RNA was analysed from brain, thymus and spleen. All thymic tumours expressed the *Thy-myc* gene at levels several-fold higher than the endogenous *c-myc* gene (Fig. 1b). Hybrid mRNA levels varied, even within a mouse line, reflecting the variability of tumour phenotype (compare lane TM25 with TM25.32 and TM25.49). Lines without tumours showed background signal (TM6L, TM7). The only tumour (TM25.49) that expressed low levels also contained mostly mature CD4⁺ single positive cells (Table 1). As reported^{11,13}, high levels of *c-myc* transgene expression reduce the endogenous *c-myc* mRNA levels (T and E in Fig. 1b). Background signal was detected in other tissues, except in the lymphoid organs which appeared enlarged in some of the animals (Fig. 1b; TM3, TM14 and TM25.120). As expected¹⁷, the transgene is switched off in cells progressing to the single positive stage (Fig. 1b, all except TM3, TM14 and TM25.120).

In the brain, unexpectedly low levels of transcription were observed, as shown by RNase protection and run-on assays (Fig. 1b, d). Expression of the *myc* gene is efficiently directed by a downstream thymocyte specific enhancer (our unpublished data), nevertheless, we have no explanation for the low levels in brain, as none of the sequences thought to be important for brain specific expression were interrupted (ref. 20; and our unpublished data).

Cell lines were established from thymomas that were of an adherent epithelial and a non-adherent thymocyte phenotype. Cultured cells were maintained in 10% fetal calf serum without any further growth factor addition. All the thymocyte cell lines are of a double positive character and express high levels of the transgene (Fig. 1c, lane *Thy*, panel *myc*). By contrast, only one out of four adherent cell lines expressed the hybrid gene at levels above background (Fig. 1c, lane D, panel *myc*).

Histological analysis also showed that both stromal and lymphoid cell populations had expanded. The tumours were composed of two main areas separated by a clear junction (Fig. 2a). Lymphocytes and macrophages were distributed throughout both areas, whereas the main blood vessels were predominantly located in the central zone. These were sometimes parts of islands of large lymphoblasts and macrophages surrounded by bands of expanded epithelial cell populations (Fig. 2a, b). The lymphoblasts were larger than normal and many mitotic figures could be seen.

Immunohistochemistry revealed that essentially all lymphoid populations were *Thy-1.2*⁺, although the intensity of the *Thy-1.2* labelling varied considerably in some tumours. The majority also stained positively for both CD4 and CD8 (data not shown). All tumours stained strongly for keratin in the central zone (Fig. 2b), whereas the outer zone was essentially negative although cells with epithelial morphology were clearly visible in this region. Dual immunofluorescence with keratin and either IVC4 (ref. 21) or 4F1 monoclonal antibodies showed that some

The expanded epithelial cells, as well as the thymocytes (see below), seem to be transformed as they can establish long-term, fast growing *in vitro* cultures and cause tumours in histocompatible animals. Epithelial transformation was unexpected, as *Thy-1* appears normally to be confined to rodent lymphocytes and has only been reported to appear on thymic epithelial cells *in vitro*²⁴ (see also Fig. 1c). Possibly, *Thy-1* and, by extension, the *Thy-myc* gene are expressed transiently during epithelial maturation. Another possibility is that the epithelial cells respond to signals produced by the over-proliferating lymphocytes in these tumours and expand, thereby increasing the probability of the occurrence of a transforming event that is unrelated to the expression of *Thy-myc* gene. Complementary situations have been described

To further characterize the lymphocyte suspensions from large thymic tumours, the progeny of the TM6 and TM25 mice were analysed by FACS (Table 1) using reagents against Thy-1 and HSA (heat stable antigen) which appear early in haematopoietic differentiation²⁷⁻²⁹, the T-cell subset differentiation markers CD4 and CD8, and components of T-cell antigen receptors V β 8 and the CD3 ϵ chain which are expressed highly only later in differentiation (for review see ref. 30). Thymocytes of young transgenic animals were normal in terms of CD3, CD4, CD8 and HSA expression (data not shown). This favours the idea that the tumours arise in a population of phenotypically normal cells, although *Thy-myc* mRNA levels in these thymuses were comparable to those in fully grown tumours (data not shown). Most tumours contained mainly CD4⁺CD8⁺ cells, which is also the predominant phenotype in normal thymus³¹. On forward light scatter, however, more than 80% of the CD4⁺CD8⁺ tumour

[illegible]

cells were large blasts, whereas most of these cells are normally smaller than resting peripheral lymphocytes²⁸. Several of the tumours were mainly CD4⁺CD8⁻ HSA-low and CD3-high (for example, TM25.49; Table 1), which is normally the phenotype of a minor population of functionally mature thymocytes^{32,33}. Other tumours contained cells with the abnormal phenotype CD4⁺CD8⁺, HSA-low (for example TM6.78; Table 1) which probably result from abnormal differentiation, although they could represent the expansion of a normally very rare cell type. In tumours with both CD4⁺CD8⁻ and CD4⁺CD8⁺ cells, Southern blots derived from FACS-sorted cells of C β rearrangement suggested that both cell populations were (mono-) oligoclonal and part of the tumour (data not shown). The expression of V β 8 in some tumours was similar to that on normal thymocytes³⁴. Some tumours, however, were clearly CD3⁺ but completely V β 8⁻, whereas others showed disproportional overexpression of V β 8, indicating that these tumours were oligoclonal. This was confirmed by Southern blots with a TCR β -chain constant region probe, to detect TCR β -chain rearrangements³⁵. Compared with liver DNA, only one or two new bands are observed due to rearrangements of the C β 1 or C β 2 locus

in any given tumour (Fig. 3), indicating that the tumours were formed by clonal expansion from one or a very small number of cells.

The tumour cells were also tested for their ability to propagate in histocompatible (CBA/CA $\times$ C57BL/10)F₁ mice after subcutaneous injection. After injection of 10⁶ to 10⁷ tumour cells, subcutaneous tumours developed in 7 cases out of 8 for TM25 and in 2 out of 5 for TM6. No correlation could be made, however, between the ability to form subcutaneous tumours in F₁ mice and metastasis in the original thymoma-bearing animals (Table 1) whose lymphoid organs were invaded by CD4⁺CD8⁺ T lymphocytes.

These results indicate that oncogenic transformation has taken place at the CD4⁺CD8⁺ cell stage in a stochastic manner and after the TCR β -chain has rearranged. In some of these tumours the cells continue to differentiate to single positive cells, and in a few cases double positive cells escaped the thymus and gave rise to metastases.

Although the mechanism of tumour formation in these transgenic mice remains unknown, it is the first time that the expression of *c-myc* has been deliberately targeted in the thymus

TABLE 1 Summary of FACS analysis on tumours in TM6 and TM25 offspring

Offspring	CD4 ⁺ CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁻ CD8 ⁻	HSA ⁺	Metastasis	s.c. passage	Cell-lines	
								Lymph.	Adher.
TM6.2	40	50	5	5	0	—	ND	—	—
.3	74	18	3	4	ND	—	ND	—	—
.75	42	54	2	2	0	—	ND	+	+
.78	74	20	1	4	1	—	+	+	+
.84	90	8	1	1	5	—	+	—	+
.137	59	12	3	26	7	—	—	—	—
.140	53	29	1	17	3	—	—	—	—
.141	56	15	3	27	34	—	—	—	—
.158	90	5	1	4	63	—	ND	—	—
.159	95	3	1	2	16	—	ND	—	—
.161	86	6	2	6	44	—	ND	—	—
.163	92	3	1	3	30	—	ND	—	—
.165	71	14	2	13	29	—	ND	—	—
.166	87	9	1	3	2	—	ND	—	—
Total:						0/14	2/5	2/14	3/14
25.2	86	0	8	0	88	—	ND	—	—
.3	33	46	8	12	13	+	ND	+	—
.4	59	28	3	10	84	—	+	—	+
.5	60	18	5	16	68	+	+	+	+
.8	63	30	2	3	ND	—	ND	+	+
.9	74	20	3	3	30	—	ND	—	—
.26	80	16	2	2	80	+	ND	—	—
.32	94	3	1	1	80	—	ND	+	+
.45	76	20	2	1	93	—	ND	+	+
.48	28	46	8	16	ND	—	ND	+	+
.49	2	72	10	17	0	—	ND	—	—
.103	50	38	1	10	ND	—	—	+	+
.105	87	4	2	7	18	—	+	—	+
.106	72	8	5	14	0	+	+	—	+
.112	96	3	0	0	ND	—	+	—	—
.114	43	29	2	26	ND	—	+	+	+
.120	85	11	0	3	ND	+	+	+	+
Total:						5/17	7/8	9/17	11/17

The columns indicate the percentage of CD4⁺CD8⁺ and HSA⁺ cells. The next two columns indicate which of the animals showed metastasis, or gave tumours when passaged subcutaneously (s.c.) in histocompatible recipients. The last two columns show which of the tumours could be established in tissue culture as non-adherent (Lymph.) or adherent cells (Adher.). The cell lines were established in RPMI medium supplemented with 10% FCS, at a density of $\sim 10^7$ tumour cells per ml. No additional growth factors were added and the established cultures were maintained at a density of 10^5 – 10^6 cells per ml. Our analysis does not distinguish between normal cells and neoplastic tissue which is the result of oligoclonal expansion of a few transformed cells. It is possible, therefore, that the phenotype we describe (by FACS and Southern blot analysis) is partly due to the presence of normal cells in the enlarged thymus. Expression of cell-surface antigens was analysed by flow-cytometry on a FACS II Unit (Becton Dickinson) using monoclonal antibodies specific for mouse: CD4, CD8, Thy-1.2, CD3 ϵ , V β 8 and HSA(J11d). Antibodies were purchased from Becton Dickinson. For mouse CD4 and CD8, antibodies were directly conjugated to fluorescein (α CD8, green) or phycoerythrin (α CD4, red), therefore, no second layer antibody was required. For CD3 ϵ and HSA, anti-hamster and anti-rat antibodies were used, respectively, with a second layer antibody.

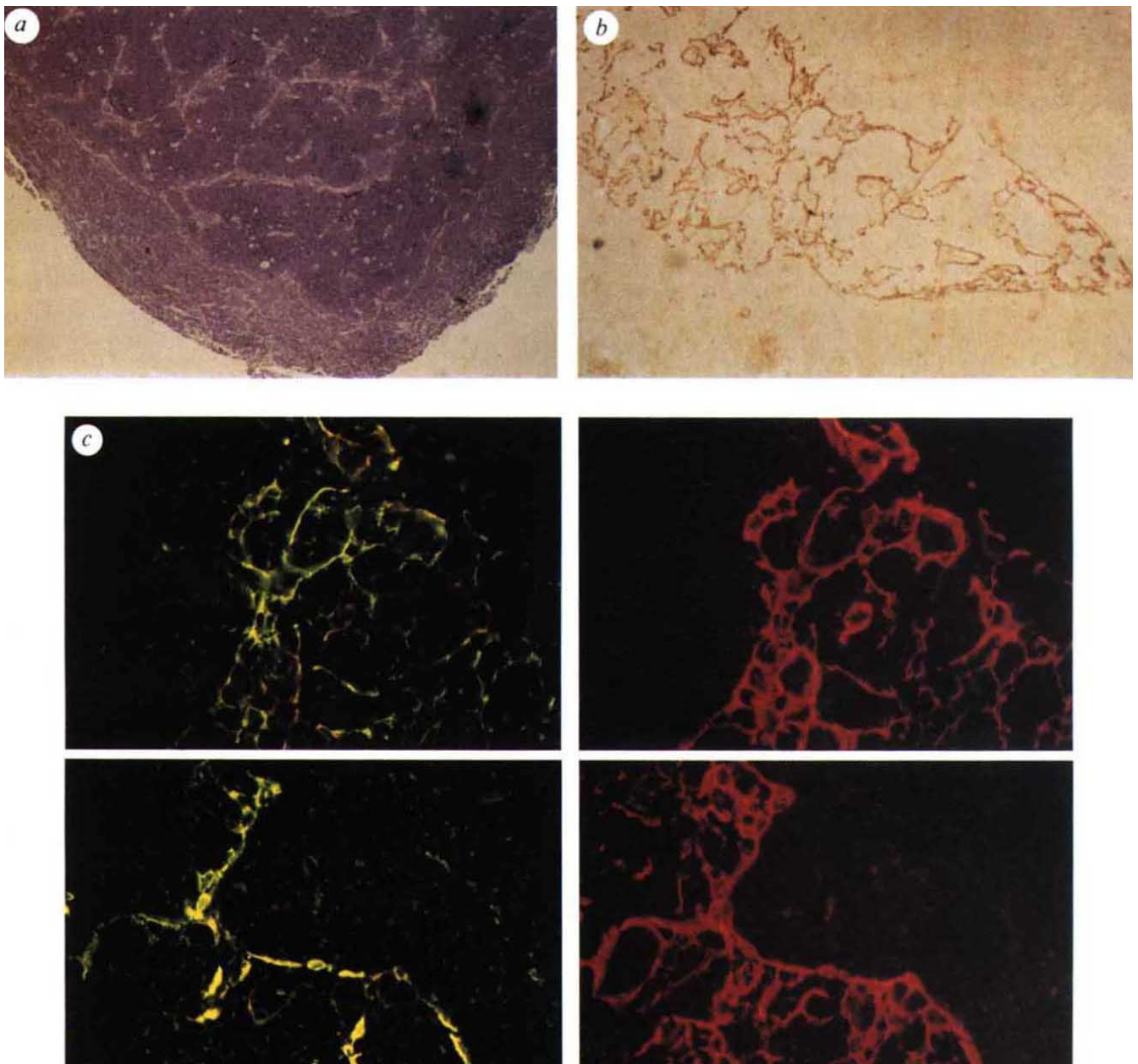


FIG. 2 Histological analysis of thymic tissues from mice TM6 and 25. *a*, TM25 tumour showing inner and outer zones. Haematoxylin stain; final magnification $\times 148$. *b*, TM25 tumour showing keratin-positive epithelial cells in the central zone. Frozen section labelled with polyclonal rabbit anti-keratin antibody by indirect immunoperoxidase technique. Final magnification $\times 370$. *c*, Dual immunofluorescence analysis of a TM6 tumour. Monoclonal antibodies 4F1 and IVC4 (green) each label a subpopulation of keratin positive (red) epithelial cells in the central zone. 1, 4F1; 2, the same field with keratin; 3, IVC4; 4, the same field with keratin. Final magnification $\times 1,850$. This TM6 immunofluorescence is a good example of most TM6 and TM25 tumours. **METHODS.** Tissue preparation. Thymuses were either snap-frozen for immunohistochemistry or fixed in buffered formalin, embedded in paraffin wax and sections stained with Haematoxylin/Eosin. Primary antibodies for immunohistochemistry were: rat IgM monoclonal antibodies 4F1 and IVC4, neat supernatant, to detect cortical and medullary type thymic epithelial cells respectively²¹; rat IgG monoclonal anti-*Thy*-1.2 (H30-12), the gift of Dr A. F. Williams, supernatant, 1:30; rat IgG monoclonal antibodies anti-CD4 (YTS191.1) and CD4 (YTS169.4) (Seralab), supernatant, 1:5; polyclonal rabbit anti-keratin, purified immunoglobulin used at 1:200 (Dakopatts). Secondary antibodies were: peroxidase conjugated swine anti-rabbit Ig, 1:20 (Dakopatts); biotinylated sheep anti-rat Ig, 1:100, species specific, (Amersham); fluorescein isothiocyanate (FITC) conjugated streptavidin, 1:100, (Amersham); rhodamine tetramethyl isothiocyanate (TMRITC) conjugated swine anti-rabbit immunoglobulin 1:20 (Dakopatts). Immunolabelling was done by standard indirect immunoperoxidase and immunofluorescence.

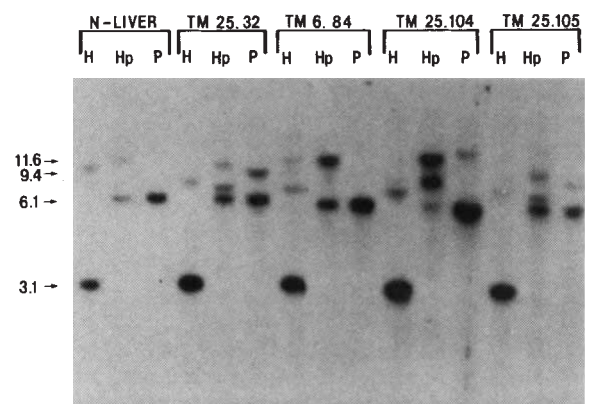


FIG. 3 Samples (10 μ g) of DNA extracted from liver or thymus were digested with *Hind*III, *Hpa*I or *Pvu*II and separated on a 0.7% agarose gel. After Southern transfer³⁸, the filter was hybridized with a TCR β constant region probe³⁹ and labelled with ³²P by the mixed primer method³⁸.

and has caused tumours of thymocyte and thymic epithelial origin. Thus, one of the important aspects of this investigation is the successful establishment of *in vitro* cultures of adherent cells with thymic epithelial phenotypes. In combination with thymocyte cultures from normal or *Thy-myc* transgenic mice, it may be possible to reconstruct *in vitro* a thymic microenvironment in order to study T-cell differentiation. □

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Inomycin-regulated phosphorylation of the myeloid calcium-binding protein p14

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TWO associated calcium-binding proteins (CaBPs) have recently been identified specifically in cells of myeloid origin. These proteins have relative molecular masses (M_r) of 8,000 and 14,000 and are variously referred to as the cystic fibrosis antigen¹, the L1 light chain², MRP-8 (ref. 3) or p8, and the L1 heavy chain², MRP14 (ref. 3) or p14, respectively. The expression of p8 and p14 seems to be confined to a specific stage of myeloid cell differentiation, because both proteins are expressed in circulating neutrophils and monocytes but not in normal tissue macrophages^{4,5}. In chronic inflammatory conditions, however, such as rheumatoid arthritis, macrophages in affected tissues express both p8 and p14 (refs 4,

6, 7). These proteins are members of a family of CaBPs of low M_r (ref. 8), which include S-100 α and β proteins^{9,10}, calyculin (2A9)¹¹, intestinal CaBP¹² and p11 (ref. 13). All the proteins have an M_r of ~10,000 with the exception of p14 which has a longer C-terminal sequence after the second calcium-binding domain. Little is known about their function, although by analogy with calmodulin they could be molecules involved in intracellular signalling that are activated by an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (ref. 14). Here we report that p14 is phosphorylated in both monocytes and neutrophils. The level of p14 phosphorylation can be increased by elevating the $[\text{Ca}^{2+}]_i$ using the ionophore ionomycin, but is not affected by activation of protein kinase C using phorbol 12,13-dibutyrate. The phosphorylated residue is threonine at position 113, which is the penultimate amino acid in p14 and contained in the longer 'tail' sequence. Part of this sequence is identical¹⁵ to the neutrophil immobilizing factors NIF-1 and NIF-2 (ref. 16), indicating that the phosphorylation event could have a role in the generation of NIF activity in the p14 protein.

We have recently described a monoclonal antibody, designated mAb 5.5, that recognizes an epitope present in p8 and p14 (refs 5, 6; J.E. *et al.*, manuscript in preparation). *In vivo* the two proteins are noncovalently associated as a heterodimeric complex, indicating that they function as a dimer (J.E. *et al.*, manuscript in preparation). We have now separated the p14 protein by two-dimensional gel electrophoresis into four distinct isoforms (Fig. 1a), all of which react with mAb 5.5 after western blotting (Fig. 1b). Thus the p14 proteins, differing slightly in apparent M_r , are characteristically present as two abundant proteins with isoelectric points (pI) of 5.4 and 5.6, and two less abundant proteins with pIs of 5.4 and 5.3. By densitometric scanning, the two most abundant forms comprise 80–95% of the p14 molecule. The detection of two principal forms of p14 is consistent with a previous observation that p14 is heterogeneous¹⁷. Because p14 is a product of a single gene, as is p8 (ref. 8), our findings indicate that some form of post-translational modification occurs, which is thought not to be glycosylation³. By contrast, the p8 protein, which was localized by analysis of the purified molecule (J.E. *et al.*, manuscript in preparation), runs electrophoretically as a single entity (Fig. 1a).

We investigated whether the p8–p14 complex is involved in signal transduction. Stimulation of myeloid cells by various factors causes an elevation of $[\text{Ca}^{2+}]_i$ and activation of protein kinase C (PKC), resulting in several specific phosphorylation events believed to be important in the regulation of complex effector functions¹⁹. We therefore assessed whether the p8–p14 complex is phosphorylated in neutrophils and monocytes after their stimulation with either ionomycin, which increases the $[\text{Ca}^{2+}]_i$, or phorbol 12,13-dibutyrate (PdBu), which activates PKC. In the absence of any specific treatment, neutrophils showed a low level of radiolabel in two proteins, corresponding to the two less abundant forms of p14 (Fig. 2a). Stimulation with PdBu for 10 min did not affect the level of phosphorylation (Fig. 2b) at any dose tested (10–1,000 ng ml⁻¹), or after shorter stimulation periods (1, 3, 5 and 7 min). By contrast, ionomycin treatment caused various increases in the phosphorylation of both p14 proteins up to 10 times the level observed in unstimulated cells (Fig. 2c). Similar results were obtained for monocytes, although only a twofold to threefold increase in phosphorylation observed after stimulation with ionomycin (Fig. 2d–f). The higher level of constitutive monocyte p14 phosphorylation could be due to cell activation caused during the purification procedure. Similar increases in phosphorylation were obtained using the calcium ionophore A23187. Under no circumstances was p8 found to be phosphorylated.

The phosphorylation site(s) in p14 was determined by phosphoamino-acid analysis and identification of a ³²P-labelled CNBr cleavage peptide. Purified p14, immunoprecipitated from ionomycin stimulated ³²P-labelled neutrophils and monocytes, was hydrolysed for 2 h and the phosphoamino acids were separ-

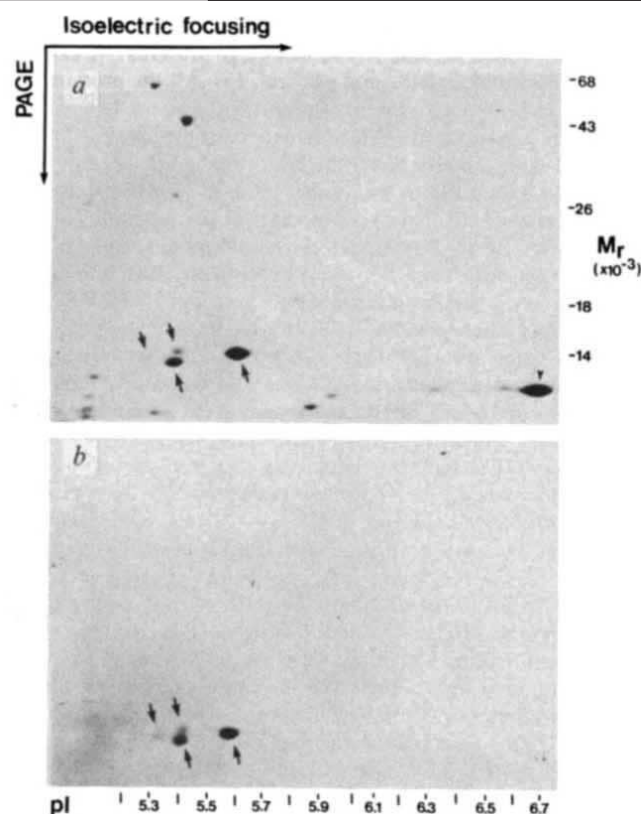


FIG. 1 Analysis of p14 in neutrophil lysates by 2-D gel electrophoresis (a) and immunoblotting probed with mAb5.5 (b).

METHODS. Neutrophils were purified by anticoagulation of fresh blood in 32 mM sodium citrate followed by incubation for 30–60 min with 6% dextran (M_r 70,000) and RPMI 1640 medium (Gibco) (1:1:1) to obtain the sedimentation of erythrocytes. To purify neutrophils from mononuclear cells and remaining erythrocytes, supernatant cells were removed, washed once in medium and separated on a discontinuous Percoll gradient as previously described²¹. All materials in contact with the cells during preparation were assayed for the presence of endotoxin using the Limulus amoebocyte assay (Sigma) and found to contain less than 20 pg ml^{-1} . Cells were prepared in this way to prevent *in vitro* activation as previously reported^{22,23}. Less than 20% of cultured neutrophils were observed to be polarized by light microscopy at the end of the purification procedure. Cells were lysed in 1% Nonidet P-40, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA containing the following enzyme inhibitors: 100 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM iodoacetamide, 50 μM soybean trypsin inhibitor, 10 μM aprotinin A and 10 μM chymostatin. Particulate matter and nuclei were removed by centrifugation at $14,000 g$ for 10 min and proteins then precipitated from the supernatant by addition of five volumes of ice-cold acetone and left for 30 min at -20°C . Proteins were pelleted by centrifugation at $14,000 g$ for 15 min and then resuspended in 9 M urea containing 1% β -mercaptoethanol. Electrophoresis of protein samples was performed by a modification of the method previously described²⁴. Briefly, proteins were firstly separated by isoelectric focusing in 10% acrylamide tube gels containing 8 M urea and ampholines spanning the pH range from 3–9. Before addition of the sample, tube gels were prefocused at 200 V for 30 min and then at 400 V for 30 minutes. Samples were then focussed for 12–16 hours at 500 V followed by 2 h at 700 V. After isoelectric focussing, tube gels were equilibrated in 125 mM Tris-HCl pH 6.8 plus 1% SDS before application onto second dimension gradient slab gels (10–20% acrylamide). After electrophoresis, slab gels were either stained with Coomassie blue for visualization of proteins or blotted onto nitrocellulose membranes as previously described⁵. Nitrocellulose membranes were incubated in a 5% milk powder solution overnight and then successively incubated for 4 h with mAb5.5 followed by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin at 4°C . The p14 proteins are indicated by an arrow and the p8 protein by an arrowhead. The peroxidase label was then developed by incubating with 3,3'-diaminobenzidine (0.6 mg ml^{-1}) and hydrogen peroxide (0.012%) for 7 min. M_r markers used were (in $M_r \times 10^{-3}$): lysozyme, 14; β -lactoglobulin, 18; chymotrypsinogen, 26; ovalbumin, 43; and BSA, 68.

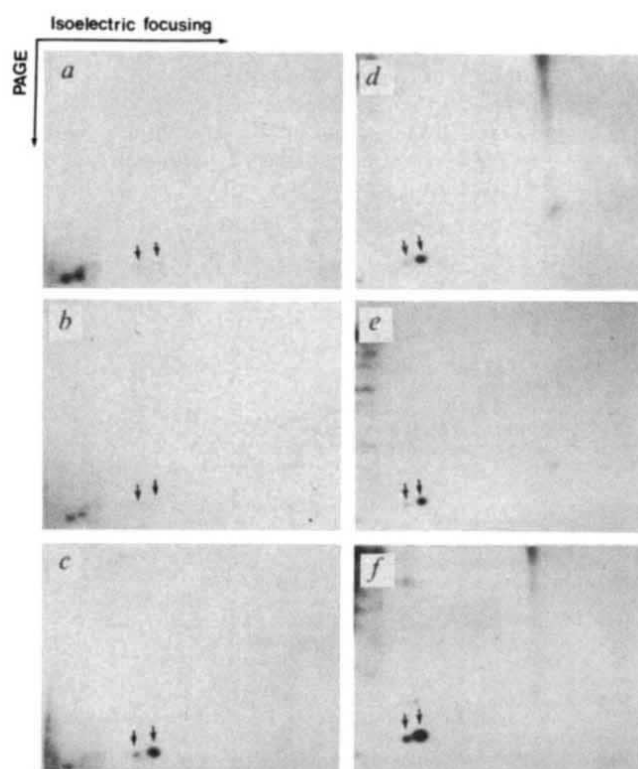


FIG. 2 Autoradiograph of 2-D gel electrophoresis of ^{32}P -labelled neutrophil (a–c) and monocyte (d–f) lysates after stimulation for 10 min with medium (a, d) 400 ng ml^{-1} phorbol dibutyrate (b, e) or 200 ng ml^{-1} ionomycin (c, f). **METHODS.** Neutrophils were purified as described in the Fig. 1 legend. Monocytes were prepared from the mononuclear cell fraction of blood obtained by Ficoll-Hypaque centrifugation and adherence to fibronectin monolayers²⁵. Before phosphorylation, cells were washed twice in RPMI minus phosphate (GIBCO) and then incubated for 90 minutes in RPMI 1640 medium minus phosphate containing 0.5% ovalbumin and 1 mCi of O - ^{32}P per 2×10^7 cells (Amersham) in Teflon beakers (Tuf-tainers, Pierce Chemical Co.). After labelling, cells were washed once and then incubated with or without the activating agents ionomycin (Calbiochem), or PdBu (Calbiochem) for 10 min at 37°C . Cells were then rapidly spun at $14,000 g$ in Eppendorf tubes, resuspended in lysis buffer and left on ice for 30 min. Lysis buffer contained 100 mM NaCl, 50 mM Tris buffer pH 7.4, 1% Nonidet P-40, 10 mM EDTA, 5 mM EGTA and the following phosphatase inhibitors; 10 mM sodium fluoride, 20 mM tetra-sodium pyrophosphate, 20 mM di-sodium hydrogen orthophosphate, 20 μM sodium orthovanadate, and enzyme inhibitors as listed in Fig. 1. Samples were then analysed by 2-D gel electrophoresis as described in Fig. 1. After electrophoresis, the gel was stained with Coomassie blue dye, destained, dried and exposed to XAR-5 film (Kodak) at -70°C . Dimethyl sulphoxide (DMSO), used to dissolve ionomycin and PdBu, did not affect phosphorylation of p14 (data not shown). To check that equivalent amounts of protein were loaded onto each gel, several Coomassie-blue-labelled proteins were scanned densitometrically. After stimulation with ionomycin or phorbol dibutyrate, alterations in the quantity of Coomassie-blue-stained abundant and less abundant forms of p14 were not readily observed by densitometric scanning.

ated by thin-layer chromatography (Fig. 3a). Threonine was the only observed phosphorylated amino acid. To determine which of the seven threonines in p14 was phosphorylated, ^{32}P -labelled neutrophil p14 was cleaved with CNBr. The resulting peptides were separated first by HPLC at pH 3.0 (Fig. 3b, top) and the fractions obtained were analysed for radioactivity. All detectable radioactivity appeared in four consecutive fractions with $>80\%$ of the counts in a single fraction (no. 25) (Fig. 3b, bottom). Protein sequence analysis revealed two peptide sequences, ARLTWASHEK (residues 84–93) and HEGDEGPGHH-HKPGLGEGTP (residues 95–114), (single letter amino-acid

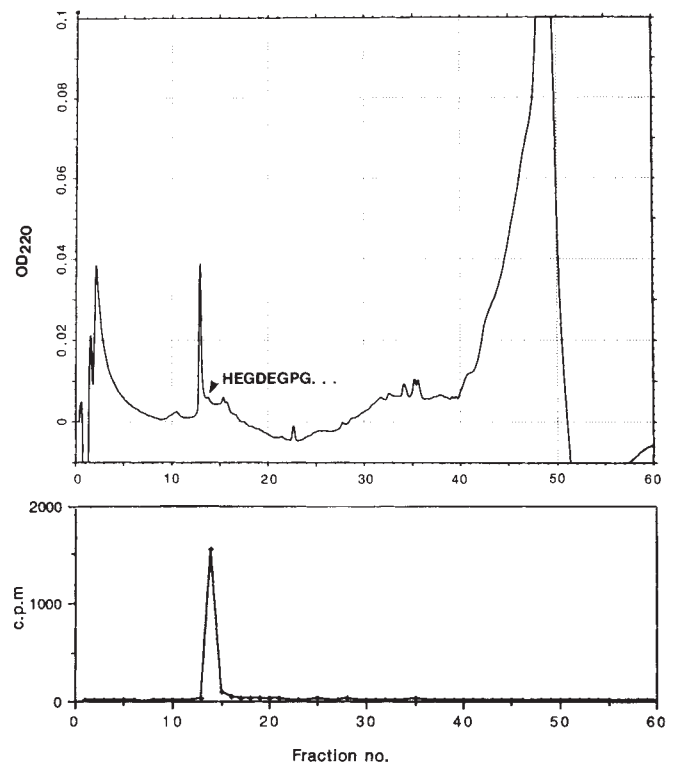
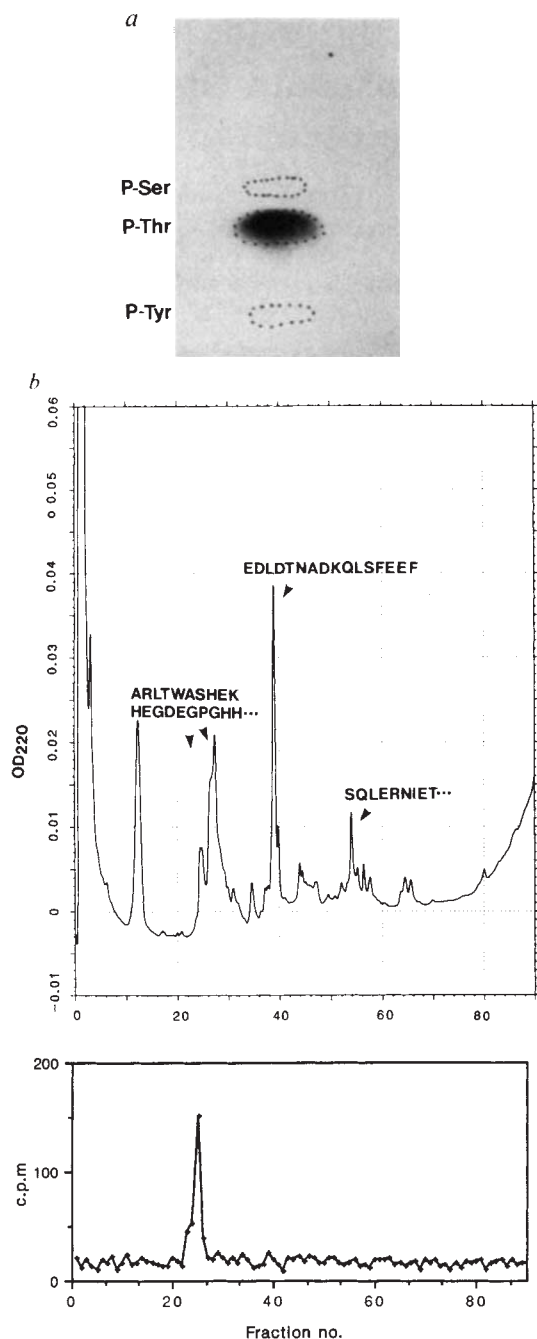


FIG. 3 Determination of the phosphorylation site of p14. *a*, Phosphoamino-acid determination of ^{32}P -labelled p14 from ionomycin-stimulated monocytes. An autoradiograph of labelled p14 phosphamino acids separated by thin-layer chromatography, with the cold marker phosphamino acids shown by dotted circles. *b*, Separation of CNBr fragments of p14 by HPLC at pH 3 showing the 220 nm absorbance trace (top) and the radioactivity in each corresponding fraction (bottom). *c*, Second HPLC separation at pH 7.0 of peptides in fraction no. 25 showing the 220 nm absorbance trace (top) and the radioactivity in each corresponding fraction (bottom). Single-letter amino-acid code is used.

METHODS. Proteins p8 and p14 were immunoprecipitated using mAb5.5 from ^{32}P -labelled ionomycin-stimulated monocytes and separated by 15% SDS-PAGE. The p14 protein was visualized by incubation of the gel in 4 M sodium acetate²⁶, excised and eluted passively into three aliquots of 50 mM ammonium hydrogen carbonate pH 8.0, 0.1% SDS, 1 mM EGTA, 0.1 mM DTT, over 48 h at room temperature. For phosphoamino-acid analysis, the eluted protein was acetone precipitated, solubilized in 6 M HCl, vacuum dried and hydrolysed at 110 °C for 2 h (ref. 27). Samples were then lyophilized and dissolved in 10 μl of 0.1 M acetic acid containing 3 μg each of phosphotyrosine, phosphoserine and phosphothreonine as standards, for separation on a 0.1-mm cellulose thin-layer chromatography plate (Camlab). The samples were separated on a flat bed electrophoresis unit (Pharmacia) for 80 min at 750 V in acetic acid-pyridine-water (10:1:189). Standards were detected with ninhydrin aerosol and the ^{32}P -labelled amino acids by autoradiography. For HPLC and sequencing procedures, pooled ^{32}P -labelled p14 was lyophilized and resuspended in 30 μl 70% formic acid in a polythene microassay tube into which had been dissolved a small crystal of CNBr. The tube was sealed, covered with aluminium foil and kept at room temperature for 16 h, then dried under vacuum and resuspended in 100 μl of 70% formic acid (pH 3.0). All the mixture was injected onto an HPLC column (Brownlee RP300) equilibrated at 0.3 ml per minute with 95% buffer A (0.08% trifluoroacetic acid and 1% acetonitrile). A linear gradient of 5–40% buffer B (0.05% trifluoroacetic acid, 90% acetonitrile) was applied over 70 min and 1-min fractions were collected. Fractions were then analysed for radioactivity by Cerenkov counting in a β -particle counter. Fractions containing radioactivity were dried down, resuspended in buffer C (1% acetonitrile, 45 mM sodium acetate, pH 7.0) and then re-loaded onto the same column equilibrated at 0.3 ml per min in buffer C, to which a linear gradient of 0–60% buffer D (80% acetonitrile, 40 mM sodium acetate) was applied over 30 min. One-minute fractions were collected and analysed for radioactivity on a β -particle counter as before. Fractions for amino-acid sequence analysis from both HPLC separations were dried and resuspended in 30 μl of 70% formic acid–10% methanol. Protein sequencing was performed on an Applied Biosystems 470A peptide sequencer with online PTH (Phenylthiohydantoin) analysis using an Applied Biosystems 120A analyser.

code), corresponding to the two C-terminal CNBr cleavage peptides of p14 (ref. 3).

To determine which of these two peptides was phosphorylated, a second HPLC separation was performed on fraction number 25 at pH 7.0 (Fig. 3c, top). Fractions eluting from the column were analysed for radioactivity, all of which was found in fraction number 14. This fraction contained the peptide HEGDEGPGHHHKPLGEGTP (Fig. 3c bottom), corresponding to residues 94–114, indicating that Thr 113 is the phosphorylation site ($n=4$). The same HPLC analysis was performed on purified monocyte p14. Although insufficient protein was available for protein sequencing, all the detectable radioactivity co-localized with the neutrophil fractions containing Thr 113. Thus Thr 113, which is the penultimate amino acid of p14, is the target phosphorylation site in both monocytes and neutrophils (Fig. 4). As only one phosphorylated sequence could be detected, we concluded that both phosphorylated p14 entities



FIG. 4 Representation of p14 showing the phosphorylated threonine residue (P-Thr 113) at the C terminus of the protein adjacent to the region found to be similar in sequence to NIF-1 and NIF-2. The two Ca^{2+} -binding domains are represented.

contained the same phosphorylated Thr 113 and that differences between the p14 variants lie elsewhere.

The results presented here indicate strongly that an increase in the $[\text{Ca}^{2+}]_i$ in monocytes and neutrophils causes a rapid phosphorylation of p14 by a kinase distinct from PKC. Whether this Ca^{2+} -dependent phosphorylation reflects the binding of Ca^{2+} to the p8-p14 complex or the activation of a Ca^{2+} -dependent kinase is, however, unclear. These results also imply that there are two main forms of p14, which differ by some unknown post-translational modification and are both phosphorylated at Thr 113 to produce the two less abundant forms. In neutrophils it has been estimated that p8 and p14 represent ~30% of the total cytosolic protein¹⁷ and in monocytes ~40-fold less than this amount (J.E., manuscript in preparation). Thus p14 is potentially a principal phosphorylated protein in myeloid cells, and this raises the possibility that phosphorylation is important for the regulation of an intracellular function. It has recently been shown that the p8-p14 complex specifically blocks casein kinase II activity²⁰. It will be interesting to discover whether the phosphorylation of p14 can regulate the inhibitory activity of casein kinase II. In addition, the C-terminal sequence of p14 is identical to peptides previously shown to have neutrophil immobilizing factor (NIF) activity^{15,16}. The connection between these two activities is unclear; but as well as having an intracellular function, the p8-p14 complex could have a role in immobilizing myeloid cells at the endothelial surface membrane both during an inflammatory response and also during normal exudation of myeloid cells into tissues, as indicated by our previous study⁵. It is possible that phosphorylation of Thr 113, which is positioned at the end of the NIF-like sequence, could have a role in the induction of this activity. □

Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor

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INOSITOL 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) serves as an intracellular second messenger for several neurotransmitters, hormones and growth factors by initiating calcium release from intracellular stores^{1,2}. A cerebellar $\text{Ins}(1,4,5)\text{P}_3$ receptor has been characterized biochemically^{3,4} and shown by immunocytochemistry to be present in intracellular membranes in Purkinje cells⁵. We show that a previously described Purkinje-cell messenger RNA⁶ encodes a protein of relative molecular mass 260,000 (260 K) with the same properties as the cerebellar $\text{Ins}(1,4,5)\text{P}_3$ receptor. Its sequence is partially homologous to the skeletal muscle ryanodine receptor⁷. By immunocytochemistry and electron microscopy the protein is shown to be present in all parts of the endoplasmic reticulum, including those that extend into axon terminals and dendritic spines. Our results indicate that gated calcium release from intracellular stores in muscle and Purkinje cells uses similar calcium-channel proteins localized in analogous intracellular compartments. This implies that the intracellular calcium stores in the endoplasmic reticulum of neurons extend into presynaptic terminals and dendritic spines where they may play a direct role in regulating the efficacy of neurotransmission.

The complementary DNA clone PCD 6035 was identified in a library of normal mouse cerebellar cDNA by its failure to hybridize to mRNA sequences from the cerebella of Purkinje-cell-degeneration (*pcd*) mice⁶. The PCD 6035 mRNA is localized to cerebellar Purkinje cells and contains an open reading frame of more than 500 amino acids (referred to as PCD6; ref. 6). We analysed the hydrophobicity profile of the derived amino-acid sequence of PCD6 and found, in contrast to the previous report⁶, the presence of at least four putative transmembrane regions (data not shown), raising the possibility that PCD6 may constitute a cell type specific channel. We next investigated the properties of PCD6 using a peptide-antiserum against the 19 C-terminal residues of its open reading frame. On immunoblots of tissue homogenates, the antibody specifically reacted with a membrane protein of ~260 K. Immunolabelling of this protein was abolished by peptide competition and was observed in rat, mouse and bovine samples, indicating a high conservation of its cognate sequence. The protein was highly enriched in cerebellum, but significant levels could also be detected in all other tissues tested, especially in liver and testis. In agreement with the large apparent molecular mass of the protein recognized by the PCD6 antibody, an oligonucleotide complementary to the PCD6 nucleotide sequence labelled a single message of 10 kilobases (kb) in RNA blots of all tissues tested (data not shown).

The affinity-purified peptide antibody was used in immunocytochemical experiments to localize PCD6 in rat cerebellum. By immunofluorescence the protein was found to be highly concentrated in Purkinje cells (Fig. 1a) and to be present in all of their cellular compartments with the exception of the nucleus, being in the perikaryon, the proximal and distal dendrites including the spines (Fig. 1e), and the axonal arbor

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(Fig. 1*b, c*). The widespread distribution of the PCD6 protein within Purkinje cells resembled that of cyclic GMP-dependent protein kinase, a soluble Purkinje cell marker⁸, except that PCD6 had a more patchy distribution consistent with its localization in a membranous compartment. Strong PCD6 immunoreactivity was also observed on ectopic Purkinje cells⁸ scattered in the cerebellar medulla, the deep cerebellar nuclei, the dorsal cochlear nucleus and adjacent brain stem regions (Fig. 1*d* and data not shown). Lower levels of immunoreactivity were found in the cartwheel neurons, a population of cells in the cortical region of the dorsal cochlear nucleus⁹. These neurons have been previously shown to share important anatomical and biochemical similarities with Purkinje cells and to undergo the same

modification as Purkinje cells in several murine mutations affecting cerebellar development, including the one used for the identification of clone PCD 6035 (refs 10, 11). Outside of the cerebellum and the dorsal cochlear nucleus, low levels of immunoreactivity were detected in selected neuronal subpopulations throughout the brain, for example in medium spiny neurons of the caudate, pyramidal neurons in the CA1 region of the hippocampus and subpopulations of cortical pyramidal cells. In testis, PCD6 was found to be concentrated in spermatozoa and their precursor cells. Although PCD6 could be detected on immunoblots in a variety of tissues tested, its expression is much lower than in Purkinje cells and generally undetectable by immunofluorescence.

FIG. 1 Localization of PCD6 in frozen sections of rat cerebellum by immunofluorescence. *a*, Sagittal section of the cerebellar cortex. Small arrows in the granule cell layer (GL) point to segments of immunoreactive axons which represent recurrent collaterals of Purkinje cell axons. *b*, Meshwork of immunoreactive Purkinje cell axons in the deep cerebellar nuclei. The neuronal perikarya (n) which are innervated by Purkinje cell axons are unstained. *c*, An arrow points to an axon emerging from one of the two Purkinje cell perikarya visible in the field. *d*, Sagittal section of the dorsal cochlear nucleus. A brightly fluorescent ectopic Purkinje cell is visible under the ependymal surface. A lower degree of fluorescence is visible in a population of neurons located in the cortical region of the nucleus, the so-called cartwheel neurons⁹ (arrowheads). *e*, High magnification of a portion of the dendritic tree of the ectopic Purkinje cell shown in *d*. The brightly immunoreactive spines are more easily visible in these ectopic cells because they stand out over a dark background.

METHODS. A peptide corresponding to residues 482–500 of PCD 6035 (ref. 6) was synthesized with an aminoterminal cysteine. Polyclonal antibodies were raised in rabbits against the peptide coupled to keyhole limpet haemocyanin²¹ and affinity purified on immobilized peptide²². Immunofluorescence was performed on 10–15- μ m thick frozen sections of 4% formaldehyde fixed rat brains⁸.

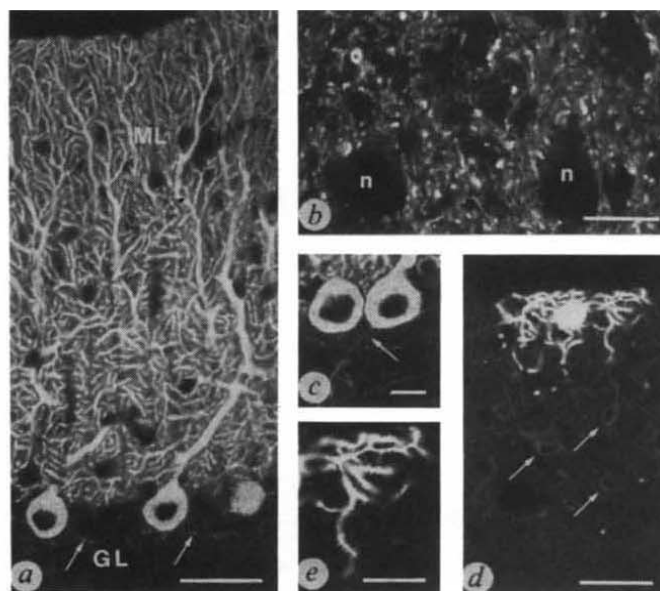
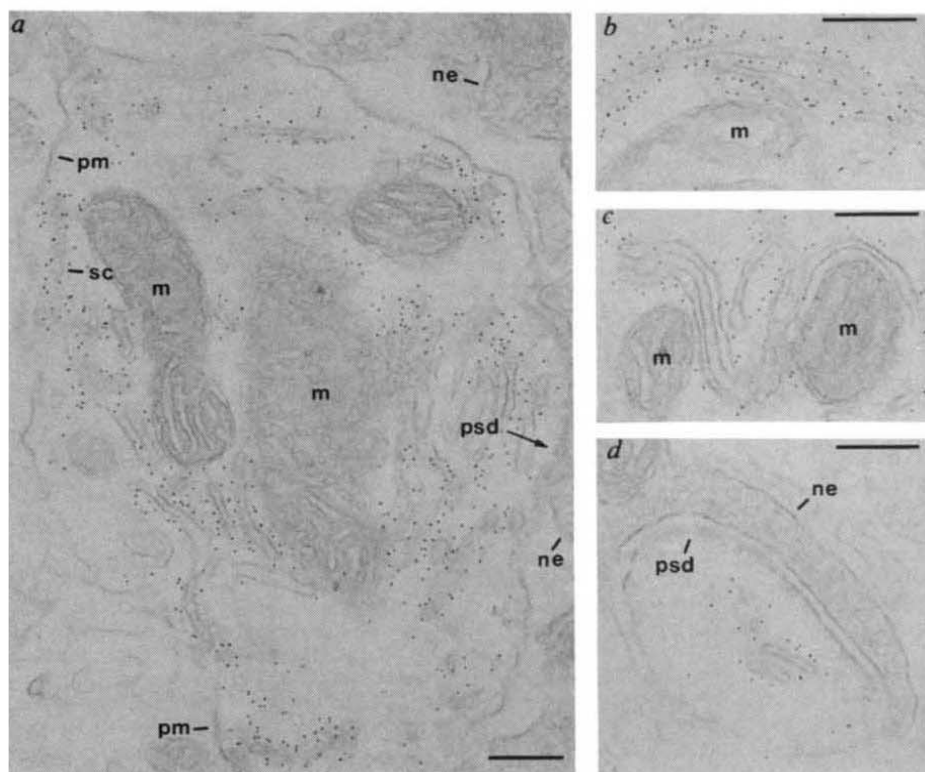


FIG. 2 Localization of PCD6 in cerebellar Purkinje cells by immunoelectron microscopy. *a*, Purkinje cell dendrite. Immunogold is concentrated on most cisternae and tubules of the endoplasmic reticulum present in the field with no labelling on the mitochondria (m) or the plasmalemma (pm). A group of cisternae is visible closely adjacent to a postsynaptic density (psd). ne, Nerve ending; sc, subplasmalemma cisterna. *b*, Labelled cisternae of endoplasmic reticulum adjacent to an unlabelled mitochondrion (m). *c*, Labelled stacks of cisternae closely apposed to mitochondria (m). *d*, Presence of immunogold on small stacks of endoplasmic reticulum in a dendritic spine (spine apparatus). The spine is surrounded by an unlabelled nerve ending (n); psd, post-synaptic density.

METHODS. Rat cerebellum was homogenized in 0.25 M sucrose, 25 mM KCl, 10 mM sodium phosphate (pH 7.4) by three up and down manual strokes in a glass-teflon homogenizer. The homogenate was immediately fixed by adding it to a large volume of 3% formaldehyde, 0.25% glutaraldehyde, 0.25 M sucrose, 5 mM sodium phosphate (pH 7.4). Subcellular particles were recovered by centrifugation, embedded in agarose, immunolabelled by a Protein A-colloidal gold procedure and processed for electron microscopy^{12,13}.



The subcellular distribution of PCD6 in Purkinje cells was analysed by immunogold labelling of agarose-embedded fragments of rat cerebella (Fig. 2) and of ultrathin frozen sections (data not shown). The agarose fragments, obtained by mild homogenization of the tissue, allowed an optimal labelling of intracellular membranes due to the dilution of cytosolic proteins^{12,13}. Both techniques revealed a dense accumulation of immunoreactivity exclusively on intracellular membranes in the shape of tubules and cisternae that were present in all Purkinje cell compartments. This indicates a localization of PCD6 throughout the endoplasmic reticulum which, in neurons, is known to extend both into dendrites and into axons¹⁴. Labelled elements included subplasmalemmal cisternae (Fig. 2a; refs 15, 16), peculiar stacks of smooth membranes as described by Rosenbluth¹⁷ (Fig. 2b), cisternae closely apposed to mitochondria (Fig. 2b, c; refs 15, 16), membranes of the spine apparatus (Fig. 2d, ref. 18), and scattered tubulo-vesicular elements in

nerve terminals. No labelling of the plasmalemma or the mitochondria was observed.

The Ins(1,4,5)P₃ receptor, purified by Suppattapone *et al.*³, is a well characterized cerebellar protein of ~260 K that is highly concentrated in Purkinje cells⁵. It is an *in vitro* substrate for protein kinase A (ref. 4) and possibly corresponds to the Purkinje cell-specific proteins independently described by Mikoshiba *et al.*¹⁹ and Walaas *et al.*²⁰. The similar molecular weights and enrichment in Purkinje cells of PCD6 and the Ins(1,4,5)P₃ receptor raise the possibility that the cDNA CD 6035 encodes part of the Ins(1,4,5)P₃ receptor. This hypothesis is supported by the finding that PCD6 and the cerebellar Ins(1,4,5)P₃ receptor of Suppattapone *et al.*³ were similarly purified by heparin-agarose chromatography which represents the largest purification step for the cerebellar Ins(1,4,5)P₃ receptor (data not shown).

To obtain further evidence for the identity of PCD6 with the Ins(1,4,5)P₃ receptor and to investigate the structural similarity of the Ins(1,4,5)P₃ receptor with the ryanodine receptor (see below), solubilized cerebellar membrane proteins were fractionated on sucrose gradients as described for the skeletal muscle ryanodine receptor²¹ (Fig. 3). Specific binding of tritiated Ins(1,4,5)P₃ cofractionated with PCD6 at a higher density position than that observed for any other protein solubilized from the cerebellar membranes, as judged by Coomassie stained polyacrylamide gels, resulting in highly enriched Ins(1,4,5)P₃ receptor preparations with an apparent molecular mass of >1,000 K (Fig. 3). This is compatible with the Ins(1,4,5)P₃ receptor consisting of a high molecular mass complex of several PCD6 subunits. Binding of radiolabelled Ins(1,4,5)P₃ was inhibited by unlabelled Ins(1,4,5)P₃ but not by Ins(1,4,5,6)P₄, indicating that the partially purified binding protein is not non-specifically binding polyphosphoinositides.

Finally, cerebellar membrane proteins solubilized with 1% Triton X-100 were incubated with 50 nM tritiated Ins(1,4,5)P₃ in the presence or absence of unlabelled Ins(1,4,5)P₃. Then the ability of the PCD6 antibody or the preimmune serum to precipitate radiolabelled Ins(1,4,5)P₃ with PCD6 was investigated. Significant precipitation of radiolabelled Ins(1,4,5)P₃ was only observed with the immune serum in the absence of unlabelled competitor, but not with preimmune serum or in the presence of a 200-fold excess of unlabelled Ins(1,4,5)P₃ (data not shown).

Accordingly, a previously described Purkinje cell-specific cDNA probably encodes the Ins(1,4,5)P₃ receptor. We observed a more widespread distribution of the putative Ins(1,4,5)P₃ receptor in the endoplasmic reticulum than reported by Ross *et al.*⁵ and our results demonstrate the presence of Ins(1,4,5)P₃ receptor containing elements of the endoplasmic reticulum both pre- and postsynaptically close to the active zone. The differences between our results and those of the previous study⁵ might be

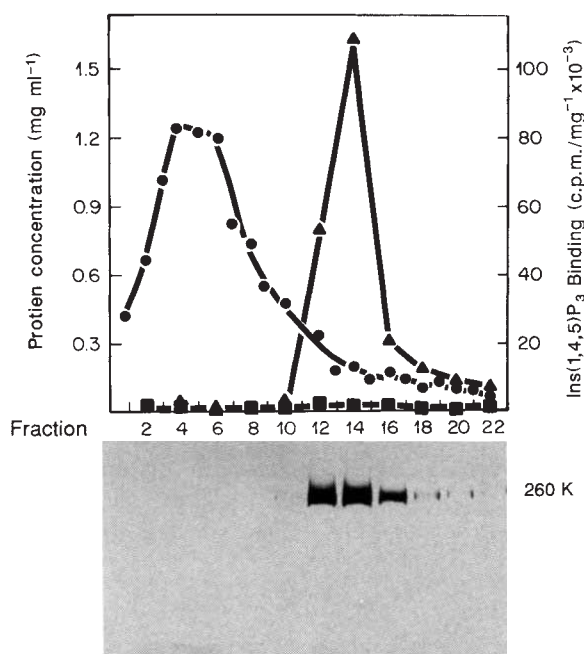


FIG. 3 Copurification of PCD6 and the Ins(1,4,5)P₃ receptor on sucrose density gradients. The graph demonstrates the distribution of total protein (circles), specific Ins(1,4,5)P₃ binding (triangles) and nonspecific binding (squares) in a 5% to 20% sucrose gradient. The immunoblot shows the distribution of PCD6 in the same fractions which precisely coincides with the specific Ins(1,4,5)P₃ binding. Analysis of the protein patterns in the same fractions demonstrated that no protein other than PCD6 first appears at the high density at which specific Ins(1,4,5)P₃ binding is observed (data not shown). Protein contaminants observed in the PCD6 fractions of the gradient were the trailing ends of abundant smaller molecular weight complexes.

METHODS. Cerebellar microsomal membrane proteins were solubilized in 1.8% CHAPS, 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 1 mM PMSF and 3 mg protein was separated on 12 ml 5–20% sucrose gradients in the same buffer at 116,000g_{max} for 16 hrs. Fractions of 0.5 ml were analysed for PCD6 immunoreactivity by immunoblotting²³. Ins(1,4,5)P₃ binding was determined in the presence of 50 nM tritiated Ins(1,4,5)P₃ (DuPont, NET-911), nonspecific binding under the same conditions in the presence of 5 μM unlabelled Ins(1,4,5)P₃ (ref. 3). Parallel gradients were run in which different molecular weight markers were added to the solubilized cerebellar membrane proteins, indicating the following peak fractions for the following markers: Bovine serum albumin (68 K), fraction 2; Alcohol dehydrogenase (150 K), 2.5; Amylase (200 K), 4; Catalase (250 K), 5; β-galactosidase (540 K), 6.5; Ins(1,4,5)P₃ receptor and PCD6, 13. Molecular weight markers are as described²⁴ (Sigma), and cold inositol phosphates from Boehringer Mannheim. In some fractions the competition of Ins(1,4,5)P₃ binding by Ins(1,4,5)P₄ was also determined and always amounted to less than 10%.

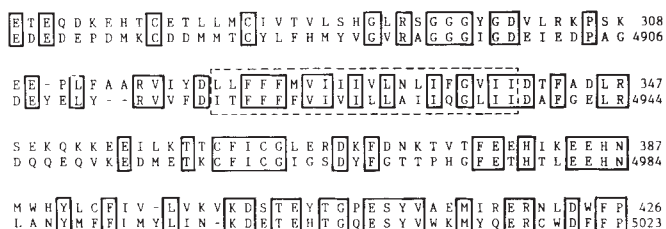


FIG. 4 Sequence alignment of the deduced amino-acid sequences encoding the putative Ins(1,4,5)P₃ receptor transcript PCD 6035 from mouse (upper line; ref. 6) and the rabbit ryanodine receptor (lower line; ref. 7). Amino acids are represented in single letter code. Identical residues are boxed with solid lines and the location of the predicted transmembrane region is boxed with a broken line. Residue numbers are shown on the right. No sequence homology was observed at the amino acid or nucleotide level outside of the region shown.

related to the different immunocytochemical technique used. Our technique was designed to maximize antibody accessibility to cytoplasmic surfaces of membranes (Fig. 3).

The skeletal muscle ryanodine receptor shares with the $\text{Ins}(1,4,5)\text{P}_3$ receptor the functional property of gating Ca^{2+} release from intracellular stores, although the two receptors are triggered by different mechanisms^{1,21}. The ryanodine receptor, like the $\text{Ins}(1,4,5)\text{P}_3$ receptor, is localized to the endoplasmic reticulum or its specializations. The ryanodine receptor is thought to be a tetramer with four transmembrane regions in each subunit that presumably form the gated calcium channel^{17,21}. PCD6 is also part of a high molecular weight complex. It migrates as very high molecular weight complex on sucrose gradients after solubilization with CHAPS (Fig. 3), and it can be crosslinked by low concentrations of the homobifunctional reagents dimethyl suberimide and disuccinimidyl tartarate (data not shown). We therefore analysed the PCD6 sequence for similarities with the ryanodine receptor sequence. Hydrophobicity plots of the PCD6 sequence indicate the presence of at least four transmembrane regions, compatible with it being a

channel protein. Alignment of the derived amino-acid sequences of the ryanodine receptor⁷ and of PCD6 reveals a strong similarity between parts of the sequences, including one putative transmembrane region (47% identity over 136 amino acids; Fig. 4). No homology was observed outside the sequence region shown, including the putative three other transmembrane regions in the ryanodine receptor and the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Because at least a portion of the region of the ryanodine receptor that is homologous to PCD6 is thought to be part of the calcium channel, our observations indicate that the calcium channel is an integral part of the $\text{Ins}(1,4,5)\text{P}_3$ receptor protein.

This study allows two conclusions regarding the release of calcium from intracellular stores. First, it shows that similar calcium channels may be used in otherwise dissimilar proteins triggered by different molecular signals. Second, it supports the concept that the endoplasmic reticulum has an analogous function in regulating cytosolic Ca^{2+} levels in neurons and muscle cells. The study indicates that this property of the neuronal endoplasmic reticulum also applies to the regions that extend into dendritic spines and axon terminals. □

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The *bcl-2* gene encodes a novel G protein

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LITTLE is known about the biochemical or functional nature of the proteins encoded by the *bcl-2* gene, which undergoes chromosomal translocation in approximately 85% of follicular lymphoma, 20% of diffuse large cell lymphoma and 10% of chronic lymphocytic leukaemia of B cells^{1–5}. Translocation of *bcl-2* sequences⁵ from chromosome 18 to the J_H segment of the immunoglobulin gene at chromosome band 14q32 in B cells results in deregulated expression of this gene, causing high steady state levels of *bcl-2* messenger RNA². DNA sequence data indicate that *bcl-2* encodes two proteins by virtue of alternative splicing, designated as *Bcl-2 α* and *Bcl-2 β* , with relative molecular masses of 26,000 and 22,000 respectively¹. Cell fractionation experiments indicate that the *bcl-2 α* gene product is located at the inner surface of the cell membrane, suggesting a possible role in mitogenic signal transduction⁶. We report here that *Bcl-2 α* has GTP-binding activity and a protein sequence that suggests it belongs to the small molecular weight GTP-binding protein (G protein) family^{7–16}.

The similarity in the relative molecular mass (M_r) (20–30K) of small G proteins with *Bcl-2 α* and its possible role in intracellular signal transduction, led us to perform a sequence comparison between them. Computer analysis revealed weak similarities between *Bcl-2 α* and several small G proteins, namely Rho (31%), Ral (33%), Arf2B (31%), Smg-21 (36%), Smg-25A

(31%) and Ha-Ras (35%) (see Fig. 1a for the complete sequence comparison between *Bcl-2 α* and two other G proteins, Ha-Ras and Rho). Similar weak homologies (30–33%) were demonstrated between several small G proteins having GTP-binding consensus sites. Notably, sequence comparison revealed significant identity (Fig. 1b) of several regions of *Bcl-2 α* with different consensus regions of Ha-Ras or G_α (α -subunit of high molecular weight G proteins) involving conservative amino-acid substitutions. For example, the PO_4 -box (one of several non-contiguous GDP-GTP binding sequences) surrounding the twelfth amino-acid position from the N-terminus of Ha-Ras (residues 10–18), exhibited ~70% identity with residues 76–84 of *Bcl-2 α* . This led us to postulate that residues 76–84 of *Bcl-2 α* are a partial sequence of the PO_4 -box. Long stretches of amino-acid residues (5–27 in Ha-Ras or 35–57 in G_α) have been postulated to be phosphate binding loops by X-ray crystallography, but so far point mutation studies have indicated residues 10–16 in Ha-Ras are most important for the binding of guanosine phosphates^{7,16,17}.

We investigated GTP-binding activity in cellular extracts from human pre-B cells (697, 380) and mouse pre-B cells (ABC-1, LS8-T2, ABE) known to express *bcl-2* (ref. 18), and NIH 3T3 cells expressing a transfected *bcl-2 α* gene (B-3, B-4 clones). We consistently detected a 26K protein which bound GTP in all of these cells (Fig. 2a, lanes 3–9). The binding of labelled GTP was performed after denaturation on SDS-PAGE gels^{19,20}, as only low molecular weight G proteins exhibit binding under these conditions and can therefore be differentiated from high molecular weight G proteins. Specificity of GTP binding was demonstrated by competitive inhibition studies using cold adenine and guanine nucleotides (Fig. 2a, lanes 10–13; data shown for 380 cell-line only). The dose response curve indicated that 1 mM guanine nucleotide was optimal for complete inhibition of binding (data not shown).

Antipeptide antibodies (S006 and S010; see Fig. 3a) reacted with the 26K GTP-binding protein in immunoblotting experi-



FIG. 1 *a*, Sequence comparison between Ha-Ras²³, Bcl-2 α (ref. 1) and Rho1 (ref. 8). Similarities between Bcl-2 α and Ha-Ras/Rho1 are indicated by vertical lines. Boxed amino acids (single letter code) are designated to show identity between Ha-Ras and Rho1. Conserved regions of Ha-Ras and Rho-1 are indicated by shading. The C-termini are marked by asterisks. Gaps are indicated by dotted lines. *b*, Sequence similarity between Bcl-2 α , Ha-Ras and G α . G α denotes a structural model of high molecular weight G protein α -subunits (adapted from ref. 17). G-box, residues required for guanosine binding; PO₄-box, residues required for binding of phosphates; S-box, residues involved in conformational switch mechanism. Vertical lines represent similarity of Bcl-2 α with both Ha-Ras and G α , or Ha-Ras only. Boxed amino acids represent similarities between Bcl-2 α and G α only. Computer analysis was carried out using University of Wisconsin genetic analysis software package (for complete sequence comparison between G α and Ha-Ras, see ref. 17).

ments with replica western blot filters carrying lysates of human (Fig. 3*b*) and mouse pre-B cells (data not shown). The antibody specificities were confirmed using the B-4 clone (Fig. 2*d*; lanes 6, 15, 16) and β -gal/Bcl-2 α fusion protein expressed in *Escherichia coli*⁶ (data not shown).

The GTP-binding ability of Bcl-2 α protein was convincingly assessed by demonstrating [α -³²P]GTP binding on immunoprecipitated unlabelled Bcl-2 α from human and mouse pre-B-cell extracts (Fig. 2*b*, lanes 8–12). The negative result using Jurkat cells (Fig. 2*b*, lane 7) can be explained as there is extremely low Bcl-2 expression in lymphoblastoid B and T cells (data not shown). The inability of isolated β -gal/Bcl-2 fusion protein to bind GTP⁶ might be due to perturbation of the proper folding of Bcl-2 protein by the bulky β -galactosidase molecule (116K), as has been observed with human glutathione-S-transferase (26K)²¹, where the *in vitro* fusion protein was enzymatically inactive.

Also, partial inhibition (50% of control) of GTP binding of the 26K protein by the S006 antibody (not S010) and conversely, a similar inhibitory effect on antibody binding by saturation of the protein with guanine nucleotides, evokes the possibility of a partial overlap of their respective binding sites (Fig. 2*c*; data shown for 380 cell-line only). This is similar to the case of p21^{ras} (ref. 7), where antibody against the GTP-binding domain completely inhibited guanine-nucleotide binding and vice versa.

Bcl-2 α protein was purified to near homogeneity by gel filtration and ion exchange chromatography¹⁴ from the particulate fraction of 380 cells (human pre-B) which carry a t(14;18) translocation¹. As shown in Fig. 4, the 26K protein purified by gel filtration and ion exchange chromatography exhibited GTP binding as well as antibody binding ability. We also electroeluted the 26K protein band corresponding to [α -³²P]GTP binding activity, from gel slices. This electroeluted protein recognized anti-bcl-2 antibody, as shown by western blotting (Fig. 4*b*, lane 4).

The experimental evidence presented here clearly documents the GTP-binding ability of the 26K protein encoded by *bcl-2*. Preliminary observations also suggest that it may have GTPase activity. Comparison of the sequence with other G proteins reveals little homology other than GTP/GDP binding regions. Taken together, the data suggest that Bcl-2 α is a novel G protein.

Despite little sequence similarity, Bcl-2 and Ras proteins behave in similar fashion in many physiological and biochemical aspects. Both are low molecular weight G proteins attached to the inner surface of the cell membrane and both can cooperate with c-Myc in cell transformation^{7,22}. In the case of Bcl-2, however, the mechanism of activation is still unclear. One possibility is that, following antigen or mitogen stimulation, the Bcl-2 protein is activated by nucleotide exchange; the activated Bcl-2 protein may then interact with some other protein in the signal-

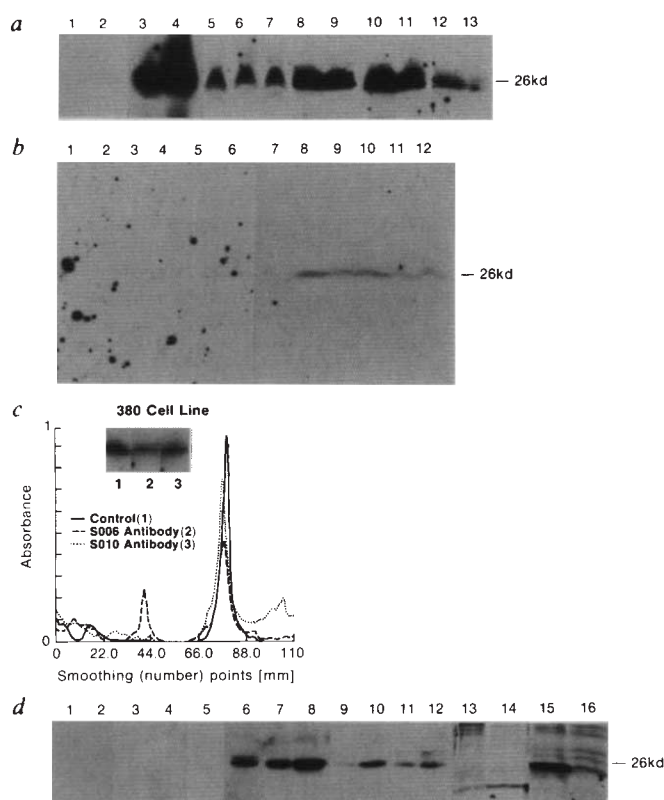


FIG. 2 *a*, [α - 32 P]GTP binding to cellular extracts of pre-B cells (human and mouse) and *bcl-2* transfected clones: effect of adenine and guanine nucleotides. Lanes 1–9, GTP binding to cellular extracts of: 490SV-1, 490SV-4 (untransfected NIH 3T3 cells), B₃, B₄ (*bcl-2* transfected NIH 3T3 cell-lines), ABC-1, LS8-T2, ABE (mouse pre-B cells), 380 and 697 (human pre-B cells), respectively. Lanes 10–13, 380 cell lysate pre-incubated with: 1 mM ATP, ADP, GTP and GTP γ S, respectively. *b*, [α - 32 P]GTP binding to immunoprecipitated Bcl-2 α from human and mouse pre-B cells. Lanes 1–6, immunoprecipitation of cellular lysates from: Jurkat, 380, 697, ABC-1, LS8-T2 and ABE cells by pre-immune serum; lanes 7–12, immunoprecipitation of cellular lysates from: Jurkat, 380, 697, ABC-1, LS8-T2 and ABE cells by S006 antibody. *c*, Inhibition of [α - 32 P]GTP binding by peptide antibody S006. Panel represents spectrophotometric scanning of the autoradiograph following [α - 32 P]GTP binding to 380 cell-line lysate pre-incubated with pre-immune serum (control), S006 and S010 antibody, respectively. *d*, Inhibition of antibody binding to Bcl-2 α protein by guanine nucleotides (shown by western blot)^{24,25}. Lanes 1–2, cellular extract from 490 SV-1 and B-4 clones, respectively, incubated with pre-immune IgG; lanes 3–4, 490 SV-1 and B-4 cell lysate incubated with S006 antibody and S006 peptide; lanes 5–6, 490 SV-1 and B-4 cellular proteins incubated with S006 antibody only; lanes 7–12, western blot using S006 antibody following pre-incubation of B-4 cell lysate with 1 mM ADP, ATP, GTP γ S, GDP, GTP and GPP (NH)₄, respectively; lanes 13–14, immunoprecipitation of [35 S]methionine labelled 490 SV-1 cells by S006 and S010 antibodies, respectively; lanes 15–16, immunoprecipitation of [35 S]methionine labelled B-4 cell lysate with S006 and S010 antibody, respectively.

METHODS. [α - 32 P]GTP binding was carried out according to established methods^{19,20}. For competitive binding studies, transfer blots were pre-incubated for 15 min with 1 μ M ATP, ADP, GTP γ S and GTP following incubation with [α - 32 P]GTP (Amersham; 3,000 Ci mmol⁻¹). For GTP-binding studies on immunoprecipitated Bcl-2 α protein, immunoprecipitation was carried out without radioisotope labelling and followed by transfer to nitrocellulose sheets (0.45 μ m). To study the inhibitory effect of antibody on GTP binding, transfer blots were pre-incubated with antibodies and then subjected to GTP binding^{19,20}. B-3 and B-4 clones were transfected with *bcl-2* DNA under the control of an SV-40 promoter by calcium phosphate precipitation technique²⁶. Metabolic labelling of 490 SV-1 and B-4 cells was carried out with [35 S]methionine (50 μ Ci ml⁻¹; Amersham 800 Ci mmol⁻¹) in methionine-free DME medium for 16 h. For both western blot and GTP-binding studies, subconfluent cultures were extracted by the method as described in Fig. 3. For immunoprecipitation, radiolabelled or unlabelled cell lysates were incubated with rabbit immune IgG or non-immune IgG at 4 °C for 4 h. Immunocomplexes were incubated with 20% IgG sorb (The Enzyme Center, Inc.) suspension at 4 °C for 2 h following extensive rapid washing of pellets with buffer containing 10 mM Tris (pH 7.4), 0.15 M NaCl, 1 mg ml⁻¹ BSA and 0.1% Triton X-100. Protein was eluted by boiling for 5 min in 0.15 M Tris-HCl (pH 6.8) containing 0.1 M 2-mercaptoethanol and 1% SDS and subjected to 15% SDS-PAGE and autoradiography.

<i>a</i>	Peptides	Sequence	Amino Acid Position
	S006	HYKLSQRGYEWDAGD	20–34
	S010	RQAGDDFSRRYRGDFAE	98–114

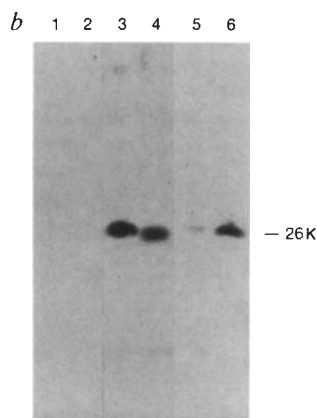


FIG. 3 *a*, Amino-acid sequence of peptide immunogen. Two peptides, S006 (amino-acid position 20–34) and S010 (amino-acid position 98–114) were chosen, from the open reading frame of the nucleotide sequence¹ of the *bcl-2* gene, to raise polyclonal antibodies in rabbits²⁷. *b*, Western blot of cellular proteins extracted from human pre-B cell-lines 380 and 697. Lanes 1, 3 and 6, 380 cell immunoblot with pre-immune, S006 and S010 IgG, respectively; lanes 2, 4 and 5, 697 cell lysate with pre-immune, S006 and S010 IgG, respectively.

METHODS. Cells ($\sim 10^7$) were collected, washed 3 times with cold PBS medium without Ca²⁺ and Mg²⁺ (GIBCO), resuspended in 200 μ l lysis buffer containing 10 mM sodium phosphate (pH 7.4), 0.15 M NaCl, 6 mM PMSF, 3% Triton X-100, 5 mM EDTA, 150 mM benzimidazole and 1 U ml⁻¹ aprotinin. Cellular proteins were extracted with repeated freezing/thawing followed by high-speed centrifugation. The clarified supernatant was used in this experiment. Equal amounts of protein (50 μ g), after boiling in 10 mM Tris HCl (pH 6.8) containing 0.1 M 2-mercaptoethanol and 1% SDS for 5 min, were size-fractionated with 15% SDS-PAGE and transferred to nitrocellulose sheet (0.45 μ m) using transblot buffer containing 25 mM Tris (pH 7.4), 192 mM glycine and 20% methanol. For western blots^{24,25}, the nitrocellulose sheets were preblocked with 5% non-fat milk²⁵ in buffer containing 10 mM Tris (pH 7.5), 0.15 M NaCl and 0.05% Tween 20, followed by incubation with antibody and ¹²⁵I-protein A (Amersham, 41 mCi mg⁻¹). Washed and dried blots were exposed to Kodak XAR-5 film for autoradiography.

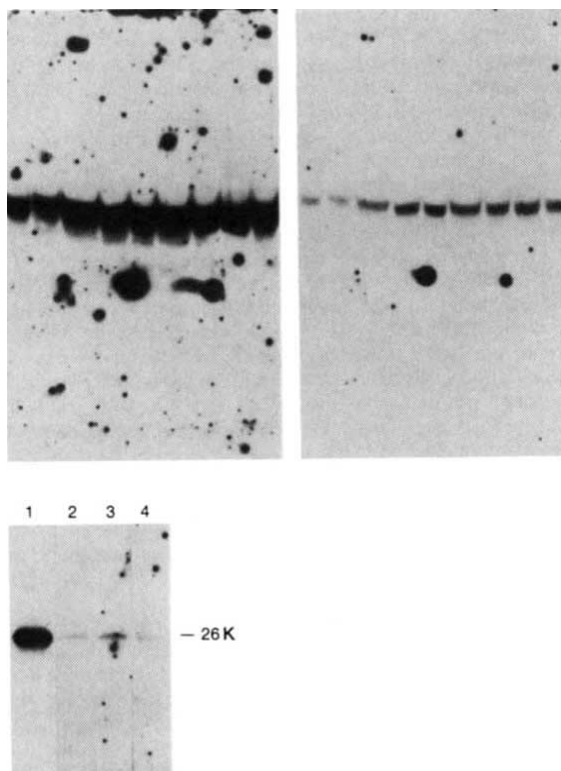


FIG. 4 Guanine-nucleotide binding activity of Bcl-2 α in purified fractions. *a*, [α - 32 P]GTP binding to different fractions eluted from an Ultrogel AcA-44 column. The left panel represents a 16 h exposure, right panel a 1 h exposure of the same blot. Note that the shorter exposure also reveals a single protein band of 26K which binds GTP. The autoradiograph is representative of fractions 21–29 eluted from the Ultrogel AcA-44 column. Besides these fractions, 16–20 and 30–34 also showed [α - 32 P]GTP-binding activity by the same molecular weight protein (data not shown). *b*, Purified as well as electroeluted Bcl-2 α protein binds [α - 32 P]GTP. Lane 1, [α - 32 P]GTP binding to a Q-Sepharose purified fraction; lane 2, western blot of the same fraction with S006 antibody; lane 3, [α - 32 P]GTP binding to electro-eluted Bcl-2 α ; lane 4, western blot of the same fraction.

METHODS. Purification of Bcl-2 protein was performed according to the method of Ohmori *et al.*¹⁴ with some modifications. Pooled fractions which showed GTP as well as antibody binding after Ultrogel AcA-44 and hydroxyapatite column chromatography, were further purified by Q-Sepharose (Pharmacia) column chromatography instead of Mono Q HR5/5 as used by Ohmori *et al.*¹⁴ A fraction of the purified protein was subjected to the above studies. For the electroelution experiment, Q-Sepharose purified fractions were run on SDS-PAGE, gel slices were cut along the 26K protein band corresponding to the [α - 32 P]GTP binding activity and the position on western blots. Biorad electroelutor (Model 422) was used for the electroelution of protein in elution buffer (25 mM Tris-HCl (pH 7.4), 192 mM glycine).

transduction pathway leading to cell proliferation. Analysis of the role of Bcl-2 in the regulation of B-cell proliferation will be an important step in understanding its role in the pathogenesis of B-cell neoplasia. □

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Association of a Ras-related protein with cytochrome *b* of human neutrophils

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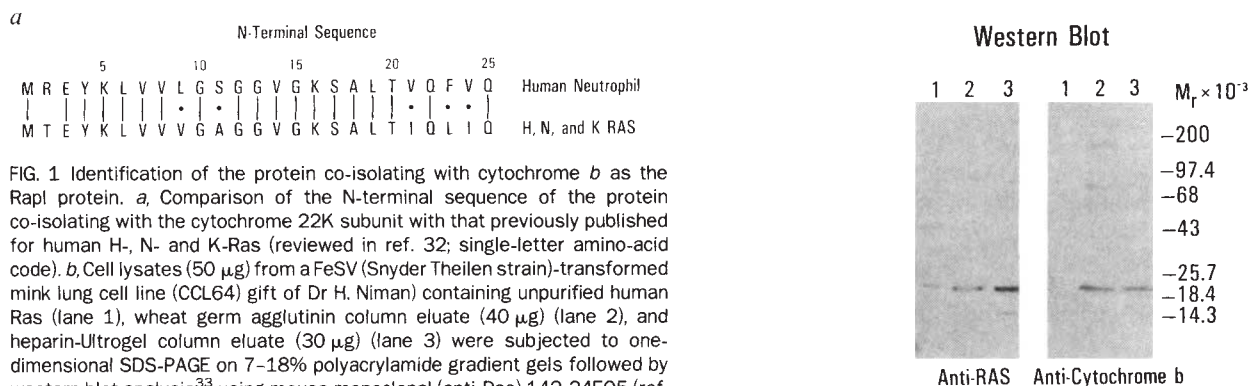
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ACTIVATION of the superoxide generating system in human neutrophils is thought to involve the interaction or assembly of cytochrome *b* with other cytosolic and membrane proteins. We have now co-isolated by conventional purification procedures a protein of relative molecular mass 22,000 with cytochrome *b*. This Ras-related protein is not a fragment of either of the subunits of cytochrome *b*, and its primary structure, as determined by the sequencing of its complementary DNA, is identical to that predicted from a recently cloned *ras*-related gene, *rap1* (also termed *Krev-1*). Immunoaffinity purification on anti-cytochrome and anti-Ras immunoaffinity matrices indicates an association between cytochrome *b* and the Ras-related protein. The association of a Ras-related GTP-binding protein with cytochrome *b* of human neutrophils could indicate a role for such a protein in the transduction, regulation or structure of the superoxide generating system.

Stimulation of human neutrophils with a variety of agents induces microbicidal responses which include the generation of superoxide anion at the cell surface^{1–3}. The plasma membrane superoxide-generating system consists of a flavoprotein NADPH-oxidase⁴, which oxidizes intracellular NADPH, thereby resulting in the transfer of electrons to a putative terminal carrier thought to be cytochrome *b* (ref. 5) and ultimately in the reduction of molecular oxygen. The rare genetic absence of cytochrome *b* in humans^{6,7}, which results in a disease in which phagocytes are unable to produce superoxide anion (that is, chronic granulomatous disease)⁸, demonstrates the essential role cytochrome *b* has in the passage of electrons to molecular oxygen.

Recent progress has been made in understanding how the superoxide-generating system is activated at the plasma mem-

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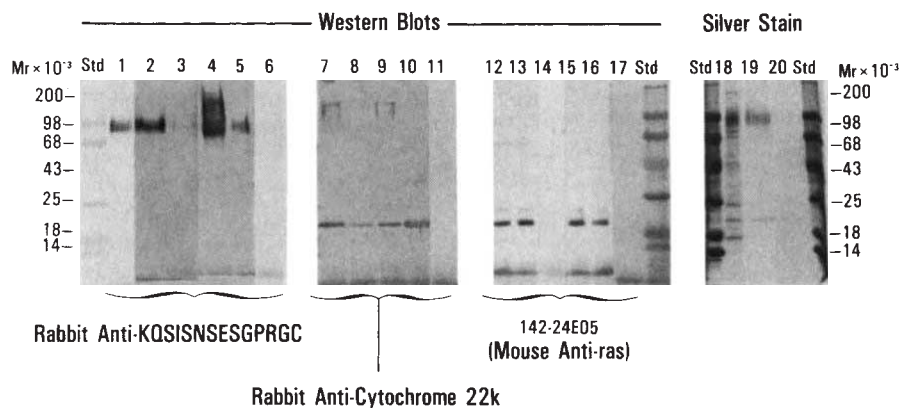
brane level. It is likely to involve the interaction or assembly of cytochrome *b* with other neutrophil proteins^{9–13}, including a phosphoprotein of relative molecular mass (M_r) 47,000 (47K)^{14,15}, NADPH-binding proteins¹⁶, flavoproteins^{4,17–20}, and GTP-binding proteins²¹. We now report the association of a Ras-related, GTP-binding protein of M_r 22 K with human neutrophil cytochrome *b*.

Human neutrophil cytochrome *b* was partially purified from stimulated human neutrophils as described²² using a multistep procedure involving: (1) exposure to high concentrations of NaCl (1 M) to remove peripheral membrane proteins; (2) octyl glucoside solubilization; and (3) lectin wheat germ agglutinin and heparin-Ultrogel affinity column chromatography. The heparin-Ultrogel column eluate was then applied to a one-dimensional SDS-polyacrylamide slab gel in preparation for amino-acid sequencing. Sequencing of the band corresponding to an M_r of 22 K revealed the presence of a double sequence (single-letter amino-acid code): GQIEWAMWANEQALASGLIL and MREYKLVVLGSGGVGKSALT. The signal generated in this sequence analysis (19 pmol) was roughly equal for

each amino acid determined. Subtraction of the cytochrome *b* light-chain sequence²³ from this double signal resulted in the latter sequence, which is very similar to H-, N-, and K-Ras proteins (Fig. 1a). Indeed, the monoclonal antibody 142-24E05, which cross-reacts with human H-, N-, and K-Ras proteins by binding to their highly conserved GTP-binding domains²⁴, recognized the co-isolating protein (Fig. 1b) in the wheat germ agglutinin (lane 2) and heparin-Ultrogel (lane 3) column eluates, as well as control samples of unpurified N-Ras from cell homogenates of a feline sarcoma virus (FeSV)-transformed mink lung cell line (lane 1).

Degenerate oligonucleotide probes coding for the N-terminal amino-acid sequence of the protein that was co-isolated with cytochrome *b* were used to obtain a cDNA clone from a human phage- λ gt10 cDNA library²⁵. The full cDNA sequence was identical to a *ras*-related cDNA, *rap1*, recently isolated from a human cDNA library by hybridization with the *Drosophila Dras3* gene at low stringency conditions²⁶, and to a cDNA clone, *Krev-1*, isolated from a human fibroblast cDNA library²⁷. We conclude, therefore, that the neutrophil protein co-isolating with

FIG. 2 Co-purification of the Ras-related protein with cytochrome *b* on immunoaffinity matrices. The amino-acid sequence determined for the heavy chain (M_r 91K) of cytochrome *b* (ref. 25) was used to determine regions of high antigenicity based on a Chou-Fasman analysis³⁴ (computer program from University of Wisconsin Computer Genetics Group). A peptide from the C-terminal region of the protein was selected, and a C-terminal cysteine was added for coupling to carrier protein. The peptide KQSISNSESGRGRC (single-letter code, residues 446–458) was coupled to KLH and injected into rabbits. After four injections of antigen during an 11-week period, antibodies were prepared as described by Doolittle³⁵. Diluted heparin-Ultrogel eluate (4.0 ml)²² of partially purified cytochrome (~0.5 nmol) was incubated for 20 h at 4 °C with 1.0 ml protein A-Sepharose (Sigma) beads covalently derivatized with rabbit anti-peptide KQSISNSESGRGRC (single-letter amino acid code; anti-KQS; lanes 2, 3, 8, 13, 14, 19 and 20), rabbit anti-cytochrome 22K subunit lanes 4, 9 and 15 (ref. 22), and mouse monoclonal 142-24E05 (ref. 24) (lanes 5, 10, 16) antibodies following the method of Schneider *et al.*³⁶. As controls for nonspecific binding, identical samples were incubated with underivatized beads, which were prepared following the same protocol but without antibodies (lanes 6, 11 and 17). The beads were washed with 10 mM HEPES buffer (pH 7.4) containing 100 mM KCl, 10 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100 and eluted with 0.05 M diethylamine (pH 11.5) containing 1 mM EDTA and 0.1% Triton X-100. The eluates were neutralized with 0.5 M Na_2HPO_4 . The heparin-Ultrogel column eluate (lanes 1, 7, 12 and 18) and immunoaffinity bead eluates were subjected to SDS-PAGE on 7–18% poly-



acrylamide gradient gels followed by silver staining (lanes 18–20)²⁸ and western blot analysis³³ with anti-peptide KQS (lanes 1–6), anti-cytochrome 22K subunit (lanes 7–11), and 142-24E05 (anti-Ras) (lanes 12–17) antibodies. To confirm specificity of the anti-KQS antibody matrix, 100 µM KQS peptide was incubated with the anti-KQS immunoaffinity beads for 10 min before adding the heparin-Ultrogel column eluate (lanes 3, 14 and 20). In addition, KQS peptide added with anti-KQS antibody during western blotting blocked all binding of the antibody (data not shown). Prestained standards were used for all gels (Bethesda Research Laboratories). Despite extensive washing of the beads during preparation, some elution of crosslinked IgG of M_r 150K was evident in the western blot analyses. The data shown are representative of five separate experiments. Std, M_r standards.

partially purified cytochrome *b* is the Rap1 (also termed Krev-1) protein, which has an overall identity of 53% to human K-Ras. We could not determine from the chromatography purification studies, however, whether the two proteins simply co-isolated or whether they were actually associated.

The possibility of a direct association of Rap1 with cytochrome *b* would be of great interest, as Ras-related proteins have not yet been shown to be associated with proteins of any known cellular function. We therefore investigated this possibility by using immunoaffinity purification studies with matrices conjugated with anti-cytochrome and anti-Ras antibodies. After the heparin-Ultrogel column eluate of partially purified cytochrome *b* was incubated with the derivatized beads, the beads were extensively washed, eluted, and the eluates analysed by SDS-PAGE followed by silver staining and western blotting (see Fig. 2 legend). The cytochrome *b* 91K subunit (lanes 2, 4 and 5), and 22K subunit (lanes 8–10), and the Ras-related protein of M_r 22K (lanes 13, 15 and 16) were present in each of the anti-cytochrome antibody bead eluates as well as in the anti-Ras antibody bead eluates, indicating an association between the Ras-related protein and cytochrome *b* even after immunoaffinity purification of the cytochrome. The same results were also obtained by using beads conjugated with antibodies prepared against a peptide determined from the amino-acid sequence of the cytochrome 22K subunit (data not shown). Silver staining²⁸ of the SDS-PAGE gel confirmed that the immunoaffinity matrices were indeed purifying the cytochrome 91K and 22K subunits (lane 19). Cytochrome heavy-chain peptide blocked the retention of most of the cytochrome *b* as well as the Ras-related protein (lanes 3, 14 and 20). As expected with any competitive binding process, a small amount of unblocked binding of cytochrome (lane 3) and Ras-related protein (lane 14) was observed. Reduced-minus-oxidized difference spectroscopy²⁹ of the eluates demonstrated that spectrally active cytochrome *b* was eluted from all three types of beads (~0.1 nmol per ml beads for each). As with the anti-cytochrome antibody beads, the anti-Ras antibody beads also retained a significant amount of cytochrome *b* (lanes 5 and 10); cytochrome *b* was not retained by undervatized beads (lanes 6 and 11). In addition, undervatized beads did not retain the Ras-related protein non-specifically (lane 17).

These results demonstrate the association of a Ras-related protein with immunopurified human neutrophil cytochrome *b*. On the basis of our biochemical sequence analysis it is most likely that this protein is the Rap1 protein. But, because the antibody used to identify it and to immunopurify its complex with cytochrome *b* was clearly not Rap1-specific, we cannot be certain that it is the Rap1 protein and not another Ras-related protein of M_r 22K. In either case, however, our results strongly indicate the existence of a molecular complex between such a protein and cytochrome *b*. This could aid the understanding of the biochemical function of Ras-related proteins and their role in the activation and production of superoxide by phagocytic cells. It is noteworthy that a recently identified M_r 47K component of this superoxide-generating system¹¹ has now been shown to have some sequence homology with GTPase-activating protein, GAP³⁰. As GAP is known to interact with *ras* p21 protein³¹, a similar association with the cytochrome-associated Ras-related protein could provide a structural link to explain the sensitivity to GTP of superoxide generation²¹. □

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Mutations that alter transcriptional activation but not DNA binding in the zinc finger of yeast activator HAP1

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TRANSCRIPTION of eukaryotic genes requires an interaction between transcription factors that bind to the TATA box region, and transcriptional activators that bind to upstream activating sequences (UASs) or enhancers^{1–6}. Several yeast upstream transcriptional activators, such as GCN4, GAL4 and HAP1, seem to contain separate domains for binding to DNA and activating transcription^{7–9}. The expression of the cytochrome genes *CYC1* and *CYC7* is controlled by HAP1, which binds to dissimilar DNA sequences in UAS1 of *CYC1* and the UAS of *CYC7*. HAP1 has a zinc-finger DNA-binding domain between amino-acid residues 1 and 148, and a highly acidic C-terminal activation domain between residues 1,308 and 1,483 (ref. 10). A mutant allele of the *HAP1* gene, *HAP1-18*, leads to a change in Ser 63 to Arg 63, immediately adjacent to the zinc finger in the DNA-binding domain^{10,11}. The *HAP1-18* mutation specifically abolishes the ability of the protein to bind to UAS1, but greatly increases the ability of the protein to activate transcription of *CYC7*^{12,13}. We now report that this increase in activation is mediated solely by the *CYC7* UAS and the HAP1-18 protein, and also that it is not caused by an altered binding affinity of the protein for the *CYC7* UAS. Furthermore, even by substituting other amino acids at position 63 and over-expressing the resulting derivatives *in vivo* we were unable to increase activity at the UAS of *CYC7* to the level obtained with HAP1-18. This rules out the possibility that the *HAP1-18* mutation increases transcriptional activation by abolishing competition by UAS1 and UAS1-like sites for the protein. We thus conclude that HAP1-18 is a better activator of transcription than the wild-type protein when bound to the UAS

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of *CYC7*. Moreover, our findings indicate that in addition to the acidic activation domain, the zinc-finger DNA-binding domain participates directly in the activation of transcription.

First we determined whether the increase in *CYC7* transcription caused by HAP1-18 is a property of the protein and its binding site alone or results from an increase in interaction with other transcriptional activators that can bind to the *CYC7* promoter. To do this a synthetic oligonucleotide (23mer) containing one or two copies of the UAS of *CYC7* was inserted upstream of the *CYC1* promoter TATA boxes, to which was fused *lacZ* as a reporter gene. This construct was then introduced into yeast strains containing either HAP1 or HAP1-18, and transcriptional activation measured by monitoring the expression of *lacZ*. HAP1-18 caused an enormous increase in *lacZ* transcription (Table 1); this increase is therefore a property of the HAP1-18 protein and its binding site, and does not require any other factors that can bind to the *CYC7* promoter. Deletion of residues 1,308-1,438, which encompass the acidic domain of HAP1-18, totally inactivated the protein (Table 1). The mutant HAP1-18 protein thus requires the acidic region to activate transcription, as does the wild-type protein.

How does the *HAP1-18* mutation lead to an increase in the ability of the protein to activate transcription? One possibility is that the mutation could increase the affinity or specificity of the protein for the *CYC7* UAS. To test for this we performed gel-shift assays using yeast extracts that contained either intact HAP1 or HAP1-18 (refs 14 and 15). For both proteins, the increase in the level of binding to a *CYC7* probe was proportional to the increase in the amount of protein present, and the levels of binding were comparable for a given amount of protein (Fig. 1a). In addition, the dissociation kinetics of the binding of HAP1 and HAP1-18 to the *CYC7* probe were indistinguishable (Fig. 1b); both proteins had partially dissociated from the probe 2 min after the addition of excess competitor probe, and by 60 min had completely dissociated. The association rates of

TABLE 1 Activity of *lacZ* (units of β -galactosidase)

	HAP1	HAP1-18	HAP1-18 Δ 1,308-1,483
CYC7 UAS single	0.1	45	—
double	0.2	441	—
CYC7 promoter	2.5	19.5	0.2

The increase in *CYC7* transcription caused by *HAP1-18* is a property of the protein and the *CYC7* UAS alone. The *CYC7* UAS GCTAATAGCGATAATAGCGAGGG, and its complementary strand were synthesized with *Xho* cohesive ends and cloned into the *Xho* site of plasmid pLG-312-178 (missing UAS1 and UAS2). Construct were characterized by DNA sequencing and plasmids with single and double UAS-containing inserts were introduced into yeast strains BWG1-7A (*MATa*, *leu 2-2,2-112*, *ura 3-52*, *his 4-519*, *ade1*) or TP25-44D (*MATa*, *HAP1-18*, *ade1*, *ura 3-52*) and the activity of the *lacZ* reporter gene assayed as described²². In the *CYC7* promoter experiment, a derivative of yeast strain BWG1-7A bearing a *HAP1* mutation and integrated *CYC7-lacZ* fusion gene with the intact *CYC7* promoter was used. This strain was transformed with a plasmid bearing *HAP1-18* (pHAP1-18 5.5), and a derivative missing residues 1,308–1,483 (*HAP1-18Δ*, 1,308–1,483). pHAP1-18 5.5 was derived by inserting a 8.0 kilobase (kb) *Cla*I-*Sma*I fragment from plasmid Ycp50-*HAP1-18*¹⁰ into a 4.5 kb backbone fragment from the 2-micron yeast origin-bearing plasmid YEp24. The deletion of residues 1,308–1,483 was made by cleaving plasmid pHAP1-18 5.5 with *Kpn*I (codon 1,308) and treating with DNA polymerase large fragment. Beta-galactosidase unit of activity are expressed as in ref. 22 ($1,000 \times [\text{OD}_{420} \text{ of cells}] / [\text{OD}_{600} \text{ of reaction} \times \text{volume of cells in ml}]$).

both proteins were also similar (Fig. 1c); partial binding had occurred within the shortest time after adding the extract to probe that we were able to measure (15 s), and association was complete by 2 min. In experiments with nonspecific competitor DNAs, we found that the specificities of HAP1 and HAP1-18 for the *CYC7* probe were also similar (data not shown). We

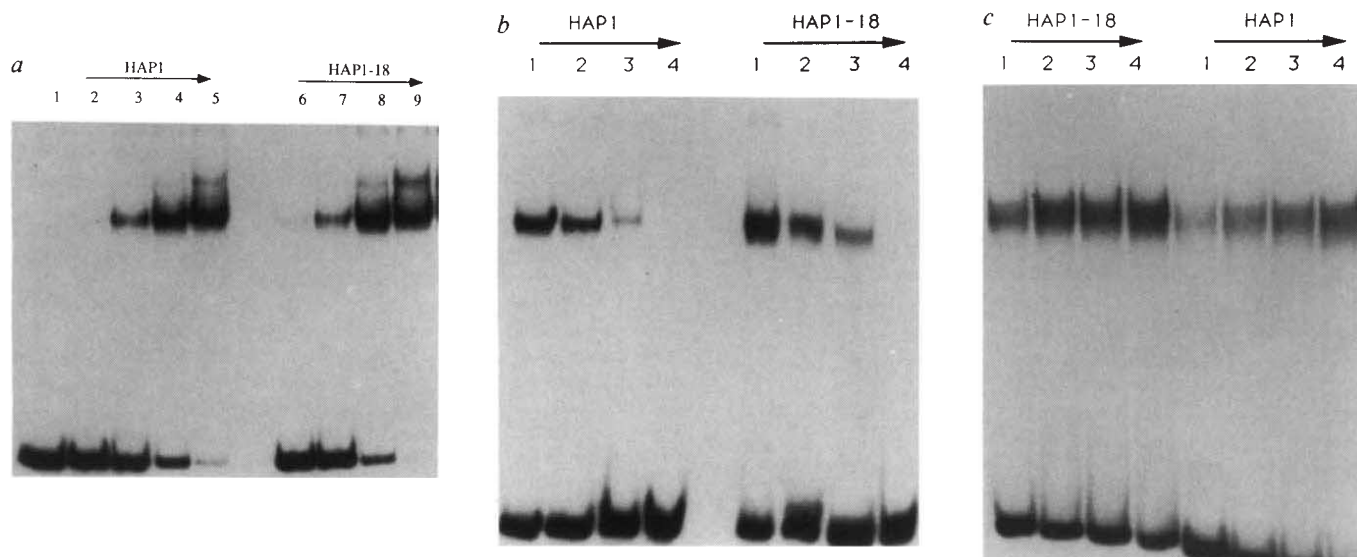


FIG. 1 Binding of HAP1 or HAP1-18 to *CYC7*. A 5.9 kb *Sma1*-*Xho1* fragment containing *HAP1-18* was excised from plasmid pSP65HAP1-18 and inserted into a plasmid pKP15 backbone cleaved with *Sma1* and *Sal1* to generate plasmid pSD5HAP1-18. The yeast strain BWG1-7A²² containing either plasmid pLGSD5HAP1 or pLGSD5HAP1-18 was grown in YEP media containing 3% raffinose to OD₆₀₀=1, and HAP induced by adding galactose to 1%. a, Extracts were prepared²² and bound to 6,000 c.p.m. of a *CYC7* probe end-labelled by filling in with nucleotides labelled with ³²P the α position) extending from -273 to -130 in the presence of 500 ng of salmon sperm DNA for 25 min. Samples were loaded on 4% acrylamide gels. The gels in this and subsequent experiments were pre-electrophoresed at 100 V for 90 min, and loaded while running. Gels were then dried and autoradiographed.

Lanes 1–5: 0, 3.8, 7.6, 15.2 or 30.4 μg protein, respectively; lanes 6–9: 3.6, 7.2, 14.4 or 28.8 μg protein, respectively. *b*, Dissociation rates, or off rates, were determined by incubating extracts with probe for 25 min, then adding excess cold *CYC7* competitor, and incubating for the indicated times before loading on the gel. Lanes 1, 0 min; lanes 2, 2 min; lanes 3, 15 min; lanes 4, 60 min. *c*, Association rates, or on rates, were determined by adding extract to probe at time 0 and stopping the reaction by adding an excess of cold *CYC7* competitor DNA at the indicated times. Addition of the competitor at time 0 completely prevented binding to the probe (not shown). Samples were immediately loaded on the gel. Lanes 1, 15 s; lanes 2, 30 s; lanes 3, 1 min; lanes 4, 2 min. Allowing association to occur for longer than 2 min did not increase the levels of bound complex (not shown).

thus conclude that the *HAP1-18* mutation does not increase the affinity or specificity of the protein for the *CYC7* UAS.

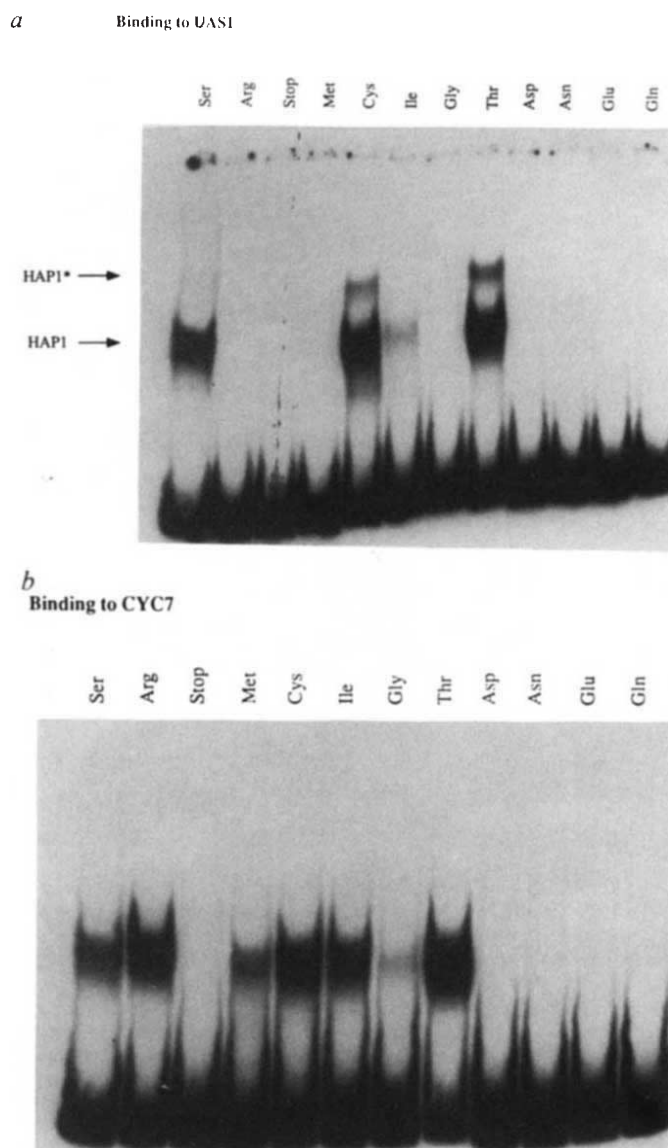
We then investigated the possibility that the *HAP1-18* mutation increases the activation of transcription by abolishing competition by UAS1 and UAS1-like sites in the yeast genome for the protein, thereby increasing the occupancy of *CYC7* UAS sites by HAP1-18 *in vivo*. We mimicked such a situation genetically by generating other changes in HAP1 that specifically abolish binding to UAS1, yet retain binding to the UAS of *CYC7*. The competition model predicts that such changes should increase expression of *CYC7*. We generated HAP1 mutant clones in which the codons for residue 63 of HAP1 were replaced with either codons for 1 of 16 different amino acids, or with UAA or UGA stop codons (Fig. 2 legend). To assess the ability of the mutant proteins encoded by these clones to bind to UAS1 and the UAS of *CYC7*, we synthesized derivatives containing the DNA-binding domain of each mutant (residues 1–245), and used these in gel-shift assays with radiolabelled probes.

Derivatives with Ser, Cys or Thr at position 63 bound to UAS1 equally well, whereas those with Ile 63 showed a greatly reduced ability to bind to the site (Fig. 2a). Those derivatives with Arg, Met, Gly, Asp, Asn, Glu or Gln at position 63 displayed no binding ability. Furthermore, derivatives with Leu, Tyr, Lys, Val or Trp were also unable to bind to UAS1 (data not shown). The complexes labelled HAP1 in Fig. 2a are those in which a protein

molecule is bound to the B-site of UAS1, whereas those labelled HAP1* are complexes in which a further molecule is bound to the A-site of UAS1 (J. Schneider, K.S.M., Pfeifer, K. and L.G., unpublished results).

We then analysed the same HAP1 mutant derivatives for binding to the UAS of *CYC7* (Fig. 2b). As with UAS1, derivatives containing Ser, Thr or Cys at position 63 bound well to the UAS of *CYC7*. In addition, derivatives containing Arg, Met or Ile at position 63 bound well to the *CYC7* UAS, whereas those containing Gly 63 bound poorly. Derivatives containing Asp, Asn, Glu or Gln (Fig. 2b), or Leu, Tyr, Lys, Val or Trp (data not shown) at position 63, did not bind to the *CYC7* UAS. Although there is no simple pattern to the amino acids at position 63 that allow binding to the UAS of *CYC7*, mutant derivatives that do not bind may have disrupted a zinc-finger structure.

All the derivatives able to bind to either UAS1 of *CYC1* or the UAS of *CYC7* were then tested for activity *in vivo*. Mutant alleles of such derivatives were recombined into yeast expression plasmids so that their expression was under the control of the *GAL* promoter¹⁶. By varying the ratio of galactose, the inducing carbon source, to glucose, the repressing carbon source, we were able to achieve a low or a high level of expression of HAP1 mutant derivatives (Fig. 2c). The *in vivo* steady-state levels of all of the derivatives that bound to the UAS of *CYC7* were indistinguishable, as judged by gel-shift analysis using yeast



C	UAS1			CYC7		
	<u>binding</u>	<u>low</u>	<u>high</u>	<u>binding</u>	<u>low</u>	<u>high</u>
Ser	+++	2.6	7.2	+++	.6	4.5
Arg	-	0.1	.2	+++	11.8	44.0
Ile	+/-	0.2	7.0	+++	0.4	8.0
Met	-	0.1	0.2	++	0.3	1.8
Gly	-	0.1	2.4	+	0.3	4.6
Thr	+++	2.5	5.0	+++	1.7	8.2
Cys	++	2.3	3.8	+++	0.5	8.3
Lys	-	0.1	0.2	-	0.1	0.2

FIG. 2 Binding and activation of HAP1 mutants. An M13 mp18 subclone containing the 350 nucleotide *Sma1-HindIII* fragment of plasmid pSP65-HAP1¹⁰ was annealed to a 25mer oligonucleotide of the sequence 5' CAAATGGTGCANNNGAGCGGAATT 3' (where N denotes an unspecified nucleotide) and filled in²³. This oligonucleotide hybridizes to the coding strand of *HAP1* and randomizes each position of codon 63. Among 35 clones that were randomly isolated and sequenced, codons for 16 amino acids (Ser, Arg, Leu, Asp, Asn, Glu, Gln, Thr, Met, Tyr, Cys, Ile, Lys, Val, Gly and Trp) as well as stop codons (UAA and UGA) were found. The DNA-binding domain (residues 1–244) of the wild-type HAP1 encoded by plasmid pSP65 and mutants were synthesized *in vitro* in a wheat germ extract¹⁰. Extracts were incubated with ³²P-labelled UAS1 (–312 to –229) or *CYC7* (–273 to –130) probes. The amino-acid change of each mutant tested is indicated, as are the HAP1 and HAP1* complexes. In *a*, the UAS1 probe was used; in *b*, the *CYC7* probe was used. *c*, Codon-63 derivatives were transferred from pSP65 HAP1 plasmids to pKP15¹⁶. The latter plasmid is a derivative of plasmid pLGS5 and contains the *GAL* promoter followed by a *Sma1* site. The plasmid also contains a *Sal1* linker inserted into the *Pvu11* site of *LacZ*, downstream of the *Sma1* site. To transfer the HAP1 mutants, a 5.0 *Sma1-Xho1* kb fragment containing the entire HAP1 coding sequence was isolated from plasmid pSP65 derivatives and inserted into a plasmid pKP15 backbone cleaved with *Sma1* and *Sal1*. Resulting plasmids were transformed into yeast strains KPY151 (MATa, *Leu* 2-2,2-112, *his* 4-519, *ade* 1-100, *ura* 3-52, dUAS2, *hap1-1*, and bearing an integrated *CYC1-lacZ* fusion under control of UAS1), and KPY152 (MATa, *leu* 2-2,2-112, *his* 4-519, *ade* 1-100, *ura* 3-52, dUAS2, *hap1-1*, and bearing an integrated *CYC7-lacZ* fusion). Beta-galactosidase was assayed as described²² in cells grown in SD minimal media supplemented with 40 µg ml⁻¹ histidine and adenine. The carbon sources were 1.2% galactose with 1.0% glucose for low expression of HAP1, or 2.0% galactose with 0.2% glucose for high expression. Also shown is a summary of the *in vitro* binding properties of the HAP1 mutant derivatives employed. Beta-galactosidase units are as in Table 1.

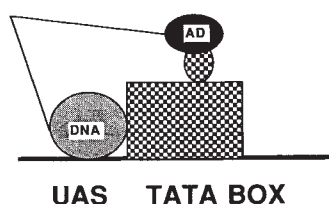


FIG. 3 A model for interactions between a transcriptional activator and the transcriptional machinery. The DNA-binding domain of the activator (DNA) bound at the UAS and the acidic domain (AD) each contact a part of the transcriptional machinery bound at the TATA box (checked). If the UAS and TATA box are not adjacent, these interactions will loop the intervening DNA. No attempt is made to distinguish among the various factors and RNA polymerase that comprise the machinery.

extracts (O. Bermingham-McDonogh and L. G., unpublished results).

The Thr-63 and Cys-63 derivatives, which bound like the wild-type HAP1 derivative to both UAS1 of *CYC1* and the UAS of *CYC7* *in vitro*, displayed activity comparable to the wild-type HAP1 derivative *in vivo*, although small deviations were seen. As expected, the HAP1-18 derivative (Arg 63) caused a striking elevation in expression of *CYC7* and did not function at UAS1. The derivative containing Lys 63, which did not bind to either of the sites *in vitro*, served as a negative control; it did not bind to either site *in vivo*.

The higher activity of the HAP1-18 derivative than the wild-type at the UAS of *CYC7* was evident even when the proteins were over-expressed. This finding argues against the *in vivo* competition model for explaining the increase in transcriptional activation.

Also relevant to the *in vivo* competition model is the behaviour of the Met-63 and Ile-63 derivatives *in vivo*, both of which *in vitro* bound well to the UAS of *CYC7*, but hardly or not at all to UAS1. *In vivo* the Met-63 derivative did not bind to UAS1 whether expressed at high or low levels, and yet the expression of *CYC7* was actually less than that with the wild-type derivative (Fig. 2c). This result therefore, also argues against the competition model; but note that the Met-63 derivative only showed a partial ability to bind to the UAS of *CYC7* *in vitro*. The activity of the Ile-63 derivative at UAS1 was conditional; when expressed at low levels the derivative showed no activity at UAS1, whereas when expressed at high levels it was as active at UAS1 as was the wild-type derivative. The ability of low levels of the Ile-63 derivative to activate the expression of *CYC7* was comparable to that of the wild-type derivative and ~25-fold less than that of the HAP1-18 derivative. Thus, under conditions in which the Ile-63 derivative does not bind to UAS1, its ability to activate transcription of *CYC7* is similar to that of the wild-type derivative and not that of the HAP1-18 derivative. When expressed at high levels, the activity of the Ile-63 derivative was comparable to that of the Thr-63 or the Cys-63 derivatives, and roughly two-fold higher than that of the wild-type derivative. The properties of the Ile-63 and Met-63 derivatives *in vivo* therefore argue strongly against the *in vivo* competition model to account for the hyperactivity of HAP1-18.

How then does the Ser→Arg 63 mutation in HAP1 result in the large increase the ability of the protein to activate transcription? Our data are most consistent with the view that the mutant is a better activator than the wild-type protein when bound at the UAS of *CYC7*. We propose that the zinc-finger DNA-binding domain works in concert with the distant acidic activation domain in the activation process. The zinc-finger structure could, through the looping out of intervening DNA, contact a component of the transcriptional machinery, such as the TATA box binding protein TFIID, or RNA polymerase, thereby bringing

the activation domain closer to the TATA box (Fig. 3). The Ser→Arg 63 mutation could strengthen this contact when the protein is bound at the UAS of *CYC7*. The acidic region of HAP1 could aid in the looping process by also contacting the transcriptional apparatus. An interaction between the DNA-binding domain of GCN4, which contains a leucine-zipper motif, and RNA polymerase II has been demonstrated *in vitro*¹⁷.

The model shown in Fig. 3 is somewhat analogous to the case of the phage-λ repressor, in which mutations in the DNA-binding domain can affect the ability of the protein to activate transcription without altering DNA binding^{18,19}. It is of interest that a mutation of a lysine to glycine immediately next to a zinc finger of the human glucocorticoid receptor abolishes activation *in vivo* with only a partial effect on binding *in vivo*²⁰. Furthermore, mutations have recently been isolated in one of the zinc fingers of the glucocorticoid receptor that allow binding to DNA but not activation²¹. The above model is consistent with the finding that fusion proteins of *lexA* and the acidic regions of other activators are functional. It is possible that the helix-turn-helix DNA-binding domain of *lexA* can also contact the transcriptional machinery. Alternatively, unlike the other acidic regions tested in the *lexA* system, that of HAP1 could be sufficiently weak that an additional contact by the zinc-finger domain is also needed.

A different model of how the HAP1-18 mutation increases transcriptional activity is that the zinc finger of the wild-type protein masks the acidic domain when the protein is bound at the UAS of *CYC7*. Arg 63, perhaps because it has the bulkiest side chain of the substitutions that allow binding to the UAS of *CYC7*, could break this interaction and increase the activity of the bound activator. Probes for the conformations of the proteins bound to the UAS of *CYC7* will be necessary to determine whether such long range effects are transmitted through the protein.

It remains to be seen how often the model of transcriptional activators with two independent domains for DNA binding and transcriptional activation will prove inadequate. An involvement of sequences in the DNA-binding domain in transcriptional activation as we have indicated presents another level at which genes could be regulated. The ability of such a domain to participate in activation could be influenced by the precise protein-DNA contacts required for specific binding. The binding of regulators, such as HAP1, to dissimilar sequences could therefore provide the proteins with additional flexibility in controlling gene expression. □

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Images of single-stranded nucleic acids by scanning tunnelling microscopy

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THE scanning tunnelling microscope^{1,2} has the potential to resolve the structure of biological molecules with atomic detail³. Progress has been made in the imaging of dried, unshadowed double helices of DNA⁴⁻⁷ and in recording images of DNA under water⁸. Also, images of unshadowed complexes of DNA with the RecA protein from *Escherichia coli* indicate that this technique may not be restricted to thin biological samples⁹. Here we present images of polydeoxyadenylate molecules aligned in parallel, with their bases lying flat on a surface of highly oriented pyrolytic graphite and with their charged phosphodiester backbones protruding upwards. Based on these images, a molecular model has been built which suggests the presence of a hydrogen bond that could stabilize the parallel alignment. Our micrographs demonstrate the potential application of scanning tunnelling microscopy in structural studies of nucleic acids and provide evidence that it could be used to sequence DNA.

Two problems hinder attempts to image unshadowed nucleic acids with the scanning tunnelling microscope (STM): low conductivity of the molecules and difficulty in adhering them to flat, relatively inert supports. In response to the conductivity question, it has been suggested that the electrons tunnel through DNA in incoherent steps, reducing the resistance of the pathway⁴. To promote firmer attachment of polyanionic molecules to a hydrophobic highly oriented pyrolytic graphite (HOPG) surface and so to prevent them from being swept away by the tunnelling tip, either the surface itself or the molecules can be modified. Denaturation of the double helix would expose the nucleotide bases, whose greater hydrophobicity would favour binding to HOPG. This might also separate the sugar-phosphate moieties from the bases, making them accessible to the tip. To avoid reannealing of the strands during the deposition onto HOPG, we have used poly(dA), which is not self-complementary. Although it is sequence-redundant, it serves to establish the mode of binding of single-stranded DNA to graphite and enabled us to investigate the possibility of using scanning tunnelling microscopy to sequence nucleic acids.

The boxed area in Fig. 1a shows parallel chains of poly(dA) in white, stretching from the lower left to the upper right. Figure 1b shows a similar array, but it is masked by noise. In Fig. 2a, the polymer chains (in blue) are spaced 10 Å apart by double-ringed structures (in orange) occurring every 6 Å along the chains. The long axes of these bicyclic structures form angles of ~50° with the chain axes. The most revealing dimension is the centre-to-centre distance in these double rings, which averages 2.40 Å. This is only slightly larger than the 2.20 Å from the centre of the imidazole ring to the centre of the pyrimidine ring of adenine¹⁰. These features are consistent with a model of parallel single-stranded DNA chains, in which the adenines stack on the benzene-like surface of the HOPG beside the polar deoxyribose-phosphodiester backbones.

Enlarged views of regions from Fig. 2a are shown in Fig. 2b and c. Notice the regular shape of the backbone features associated with two different bases in Fig. 2b (rounded arrows). In Fig. 2c, the lattice features are most regular, and the imidazole (thin arrows) and pyrimidine rings of the adenines (thick arrows) can be seen (also in Fig. 2b). The larger pyrimidine rings seem

deeper and wider than the imidazole rings and their hexagonal shapes are discernible.

Two tetranucleotide models of poly(dA) were constructed on the basis of these observations and crystal diffraction data¹⁰ (Fig. 4). All sugars were in the C_{2'} *endo* conformation. Arrangements in which the bases lie flat on the graphite with the backbone raised upwards are restricted by the chirality of C_{1'}. Modelled bases were rotated about the C_{1'}-N₉ bond to a position half-way between the *syn* and *anti* conformations. In Fig. 3, the STM image has been overlaid with the projection of a model in which the backbones are arranged to allow the scaled equivalent of a 6 Å periodicity among the bases. The axes of the bases are aligned at 50° angles with respect to the 5'-3' axis of the chain, in accordance with the C_{2'} *endo* puckering of the

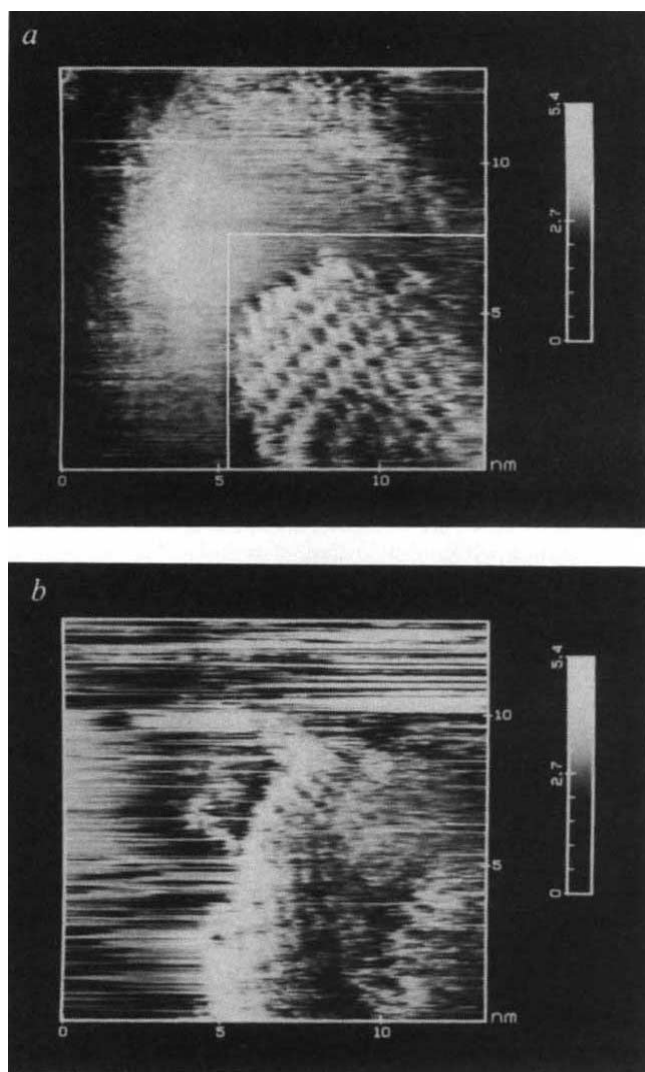


FIG. 1 a and b, Unprocessed top-view images of poly(dA) strands on HOPG. White indicates higher *z* displacements.

METHODS. One μ l of polydispersed poly(dA) ($2.2 \mu\text{g } \mu\text{l}^{-1}$) in 10 mM Tris, 1 mM EDTA, pH 8.0, at 23 °C was placed on a chip (10 mm $\times$ 10 mm $\times$ 2 mm) of freshly cleaved HOPG (zya grade; Union Carbide). The chip was surrounded by a reservoir of water and both were covered for one hour ($P_{\text{H}_2\text{O}} = 23 \text{ mm Hg}$). Ethanol (0.5 μ l) was added to reduce surface tension and the droplet was allowed to evaporate. The sample was rinsed by floating the HOPG chip on double-distilled water and the residual liquid was removed by touching one edge of the droplet with a tissue. The sample was scanned in constant current mode at 5.79 Hz with a Nanoscope II (Digital Instruments) using a 0.010 $\times$ 0.25 inch platinum/iridium (80/20) Nanotip™ (Digital) in a 1.0 μ m scan head. The bias voltage was 180 mV and the current setpoint was 0.16 nA.

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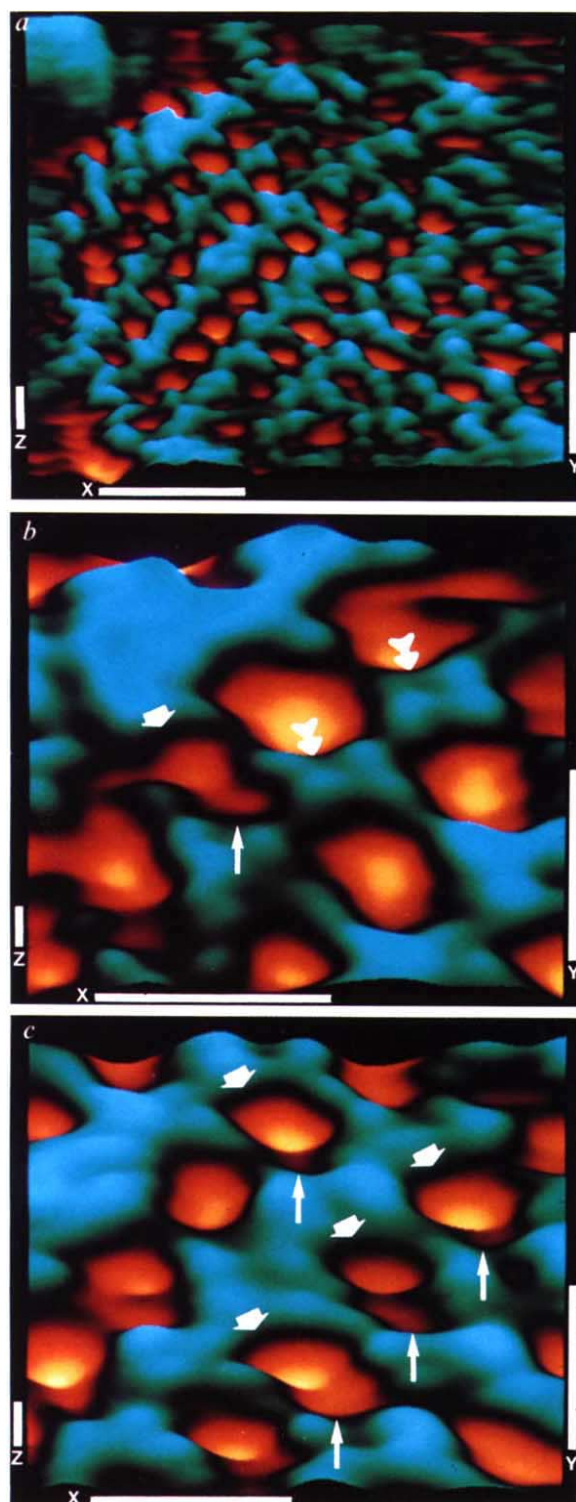


FIG. 2 All images are projections from 60° above the surface. The lowest features appear in yellow, followed by orange, black, and the highest level is in blue. *a*, Boxed area in Fig. 1*a*, low pass filtered and with axial frequencies of its Fourier transform space attenuated. Scale bars, 2 nm. *b*, Upper left region of *a*. Scale bars, 1 nm. *c*, Central region of *a*. Scale bars, 1 nm.

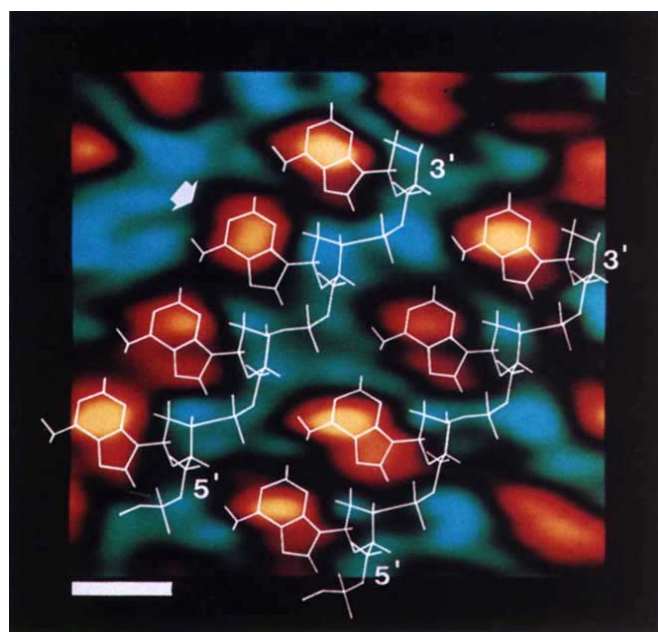


FIG. 3 An overlay of two tetranucleotide poly(dA) strands on a top view of Fig. 2*c*. Although the positions of the bases in the overlay coincide with those of the image, they seem larger in the latter, despite having equivalent centre-to-centre distances between the imidazole and pyrimidine rings. This is due to the choice of contour height for the transition from orange to blue. The apparent size of the bases can be reduced by lowering that choice at some cost to clarity. Scale bar, 0.5 nm.

sugars that determines this orientation, but note that one base (arrow) is more aligned with the backbone. In the model, adoption of the C_3' *endo* conformation by the associated pentose produces this alignment. The model also indicates a mechanism whereby the parallel chains could be stabilized. A hydrogen on N_6 of each adenine angles towards an O_4' on the neighbouring strand (Fig. 4). They are separated by 3 Å, which is within the 2.74–3.07 Å range of hydrogen bond lengths for base pairs¹⁰. Between adjacent bases, the high bias voltage and low tunnelling current, which prevented imaging of the HOPG surface, apparently created the raised divisions that are due to large tunnelling currents from the bare HOPG.

This model satisfies all but the vertical dimensions of the image. The average z displacement from the contour of a base to the peak of the neighbouring backbone is 8.4 Å, which is greater than the 5.7 Å of the model. This may stem from various tunnelling parameters that influence vertical measurements^{6,11}. Also, during the scanning from right to left, the tip may have pushed the backbone upwards, raising N_9 ; this may explain why the pyrimidine rings are deeper than the imidazole rings.

The adsorption of single-stranded DNA molecules with their non-polar bases lying flat on the surface is consistent with the hydrophobic nature of HOPG. This interaction apparently is strong enough to prevent the tip from dislodging the parallel

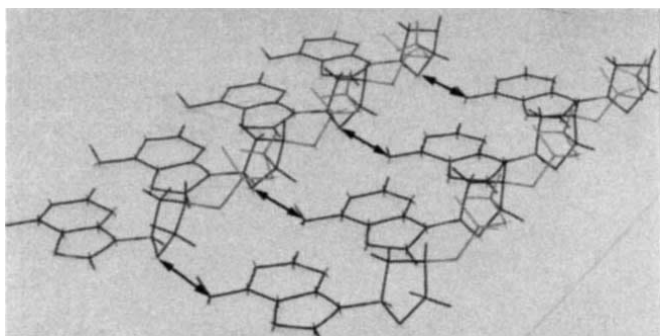


FIG. 4 A molecular model of the adsorption of poly(dA) on graphite. The planar bases lie flat beside the sugar-phosphodiester backbone. Arrows link O_4 and N_6 atoms of adjacent strands.

strands. The arrangement may be stabilized further by hydrogen bonds between the N_6 and O_4 atoms of adjacent chains.

These results show that sequencing nucleic acids by direct STM imaging is feasible if three problems can be overcome. First, formation of monolayers of single-stranded DNA with other sequences must be achieved, perhaps by adding chaotropic agents to prevent double-strand formation during deposition. Second, depositions in which the bases are completely accessible must be well controlled. Third, with the present resolution, it is only possible to differentiate purines from pyrimidines. Sequencing will require either increased spatial resolution or the specific labelling of the bases with bulky, identifiable groups. Direct sequencing would require far less material and would be more rapid than is possible at present. $\square$

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CORRECTION

Phasing of protein-induced DNA bends in a recombination complex

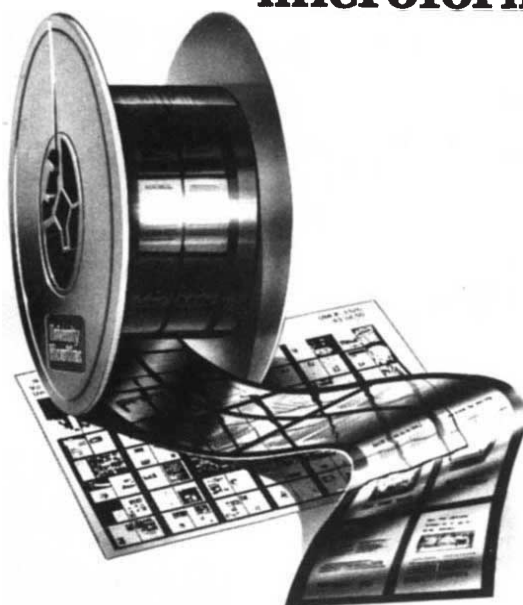
Ursula K. Snyder, John F. Thompson & Arthur Landy
Nature **341**, 255-257 (1989).

It has been brought to our attention that Fig. 4 is the mirror image of what it should be. Addition of the three-phased bends induced by IHF predicts a right-handed solenoidal coil for the path of *attP* DNA, not the left-handed coil shown in Fig. 4.

Topological analyses by Griffith and Nash¹ predict a left-handed solenoidal coil for the recombinogenic complex of Int and IHF. We are currently investigating the basis and implications of this difference. We thank Howard Nash for pointing out the reversal of handedness in Fig. 4. $\square$

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Genetic engineering of factor VIII

R.J. Kaufman

Genetic manipulation of mammalian cells has provided a means of producing unlimited quantities of a high purity, virus-free preparation of factor VIII — the most complex protein manufactured through rDNA technology to date.

HAEMOPHILIA A is a bleeding disorder that was recognized over 1,700 years ago, as documented in the Talmud. By the early 1800s, it was observed that haemophilia A is an X chromosome-linked disorder that results in a bleeding diathesis in affected males. By 1840, transfusion of whole blood was shown to successfully treat a haemophilia bleeding episode. In 1911, the presence of factor VIII in plasma was demonstrated¹, and in 1937, its crucial role in haemostasis was realized². However, a detailed biochemical and structural characterization of factor VIII has been achieved only within the last 10 years.

Current treatment of haemophilia A involves frequent infusion of preparations of factor VIII concentrates derived from human plasma. While this replacement therapy is effective in controlling many bleeding episodes, several significant problems remain. First, patients are at risk of virus-transmissible diseases such as hepatitis and AIDS. The risk of viral infection associated with treatment of haemophilia A has been greatly minimized by further purification of factor VIII from plasma using monoclonal antibodies, and by the addition of virus-inactivation steps. However, these preparations of factor VIII have greatly increased the cost of treatment.

Second, due to the limited availability of plasma-derived factor VIII, patients are generally treated episodically on a demand basis as opposed to prophylactically. A consequence of this therapeutic regimen is chronic bleeding into the joints leading to tissue damage in later life. In addition, frequent intravenous infusion of factor VIII has associated difficulties, such as reduced access to peripheral veins.

Finally, about 15 per cent of patients develop inhibitory antibodies to factor VIII. Treatment of these 'inhibitor' patients has been particularly problematic. To date, the most successful therapy is the administration of high doses of factor VIII, which elicits suppression of factor VIII antibody production. This therapeutic approach is limited by the availability of plasma-derived factor VIII.

Dramatic improvements in the therapeutic regimens for haemophilia A over the past 30 years have resulted from an increase in our basic understanding of blood coagulation. Major advances

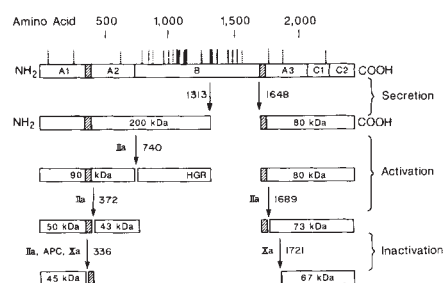


FIG. 1 Domain, structure and processing of factor VIII. Thrombin: IIa; activated protein C: APC; factor Xa: Xa; hatched areas: highly acidic regions; vertical bars: position of potential asparagine-linked glycosylation sites.

within the past 10 years include the application of modern technology to improving the purification of factor VIII from human plasma and the isolation of the human factor VIII gene.

Factor VIII is a crucial co-factor in the intrinsic pathway of blood coagulation. It accelerates the activation of factor X by factor IX in the presence of calcium and phospholipid. In plasma, factor VIII is bound by noncovalent interactions to von Willebrand Factor (vWF), which is essential for platelet adhesion to subendothelium and for ristocetin-induced platelet aggregation. As vWF also plays an important role in stabilizing and regulating factor VIII activity, the activities of factor VIII and vWF are closely associated. The relationship between factor VIII deficiency and vWF deficiency has, for many years, caused considerable confusion as the autosomally inherited von Willebrand's disease is associated with some degree of haemophilia A. Early preparations of antihemophilic factor not only corrected the slow clotting time of hemophilic plasma but also restored platelet adhesion and aggregation defects of von Willebrand's disease. The purification of factor VIII from plasma by monoclonal affinity chromatography^{3,4} has demonstrated that factor VIII and vWF are two separate proteins, which exist as a complex and which are under separate genetic control.

Factor VIII products

The application of affinity chromatography utilizing monoclonal antibodies to vWF³ or to factor VIII⁴ has yielded two factor VIII products — Monoclate

(Armour Pharmaceutical Co.) and Hemofil M (Hyland Division, Baxter Healthcare Corp.) — which have been purified from plasma about 200,000 fold. The ratio of factor VIII antigen to activity is approximately four for Monoclate and one for Hemofil M. Human albumin is added as a stabilizer to both products and there is approximately a 1:1 molar ratio of vWF to factor VIII.

In addition to monoclonal antibody purification, both products undergo an additional viral inactivation step involving either treatment with an organic solvent-detergent solution (Hemofil M), or heating (Monoclate). Both Monoclate and Hemofil M appear to carry a markedly reduced risk of virus contamination as compared with earlier preparations of factor VIII. Both products can be infused in a smaller volume, are effective, and show a normal recovery and half life *in vivo*.

Unfortunately, further purification has been accompanied by increased costs. These high-purity, high-cost products have raised an important unresolved question: whether the administration of allogenic proteins present in lower purity factor VIII preparations to patients with chronic haemophilia alters their immune function and renders them more susceptible to infectious or other diseases. If so, Hemofil M and Monoclate should minimize potential alterations to the immune system that are caused by overloading the patient with allogenic proteins.

Structure of factor VIII

A further significant advance in our understanding of factor VIII and the treatment of haemophilia A has resulted from the isolation of the factor VIII gene^{5,6} and its expression in mammalian cells^{6,8}. Our present understanding of the structure of factor VIII is shown in Fig. 1. The factor VIII heterodimer is processed from a single precursor polypeptide upon secretion from the cell. There is a metal ion-dependent association between the 80 kDa carboxy terminal-derived light chain and the 90-200 kDa amino terminal-derived heavy chain. The deduced amino acid sequence reveals three structural domains, which include a triplicated A domain of 330 amino acids, a unique B domain of 980 amino acids and a C domain of 150 amino acids. The A domains occur twice in the heavy chain and once in the

light chain and have 30 per cent homology to one another and to the A domains of ceruloplasmin — a copper-binding plasma protein — and to the A domains of factor V — a homologous coagulation factor. The C domains occur twice in the carboxy terminus of the light chain and exhibit homology to discoidins, which are phospholipid-binding proteins. The B domain is encoded by a single large exon of 3,100 nucleotides, has no homology to other known proteins, and contains 18 of the 25 potential asparagine-linked glycosylation sites within factor VIII. In plasma, factor VIII circulates as an inactive cofactor, which requires cleavage by thrombin or factor Xa at residues 376 and 1689 for activation. Proteolytic inactivation may occur through cleavage at residue 336 (ref. 9).

Expression of factor VIII

Although the hepatocyte is likely to be the cell type that produces factor VIII *in vivo*, to date, there are no known natural cell lines that express factor VIII. To obtain expression of factor VIII, the cDNA was inserted into a mammalian cell expression vector and co-transfected with a dihydrofolate reductase (DHFR) selectable genetic marker into DHFR-deficient Chinese hamster ovary cells (CHO)⁸. Cells that had a 500-fold amplification of the DHFR selectable marker gene and the factor VIII gene were selected for by increased resistance to methotrexate. Factor VIII expression in these cells was 2–3 orders of magnitude lower than that observed with other genes using similar vectors and approaches in CHO cells.

Researchers at Genetics Institute have identified three reasons for the low level of expression. First, vWF is required for the accumulation of factor VIII in the conditioned medium. Von Willebrand factor promotes association of the heavy and light chains of factor VIII upon secretion from the cell and, subsequently, protects factor VIII from proteolysis. Second, a proportion of the newly synthesized factor VIII is never transported from the endoplasmic reticulum (ER) to the Golgi apparatus. In the ER, factor VIII is found in association with an ER resident protein — the 78 kDa glucose-regulated protein (GRP78 or immunoglobulin-binding protein)¹⁰. Finally, the factor VIII mRNA level is low as a result of a post-transcriptional event.

The identification of these limiting steps for factor VIII biosynthesis in CHO cells has led to improvements in the expression of factor VIII. Factor VIII-producing CHO cells have been derived which co-express human vWF¹¹. These co-expressing cells accumulate 10 to 20-fold greater levels of factor VIII in the conditioned medium and no longer require serum as a vWF source. Reduction of cellular GRP78 levels through the use of

Medica '89 in brief

A robotic sample processor and an ELISA kit for human interleukin-6 can be seen at Medica '89 in Düsseldorf, West Germany, 22–25 November.

JANSSEN Biochimica has introduced a range of **polyclonal anti-human IgG subclass reagents** (Reader Service No. 101).



Janssen's reagents for RID.

The quantification of human IgG subclasses in serum using radial immunodiffusion techniques has, says Janssen, been difficult in the past because of the existence of allotypes. The company says that their new line of reagents, produced using advanced immunization and absorption protocols, give sharp precipitation rings with radial immunodiffusion. The reagents, says Janssen, react specifically with human IgG subclasses, show no reaction with allotype or iso-allotype determinants, and the sum of the four IgG subclasses equals total IgG. Janssen Biochimica will be represented at Medica '89 by Viro Tech GmbH on stand number E25 in hall 5.

anti-sense RNA expression has resulted in improved secretion of a protein which avidly binds to GRP78¹². This suggests that manipulation of the cell's secretory pathway may positively affect factor VIII secretion. Finally, using inducible transcription vectors, it has been possible to improve the level of factor VIII mRNA¹³.

Clinical trials

To date, two groups have entered advanced clinical trials with recombinant factor VIII (Recombinate, Hyland Division, Baxter Healthcare Corp.; Cutter Biologicals, Division of Miles, Inc.). In both cases, the factor VIII is essentially homogeneous and has a specific activity of between 4,000 and 8,000 units per mg. Detailed analysis of the structural properties of recombinant factor VIII has demonstrated it to be similar to plasma-derived factor VIII, both in the subspecies of polypeptides present and in the type and extent of asparagine- and serine/threonine-linked glycosylation. In patients, both products have been shown to be well tolerated and exhibit clearance and half-life values that are characteristic of plasma-derived factor VIII^{14,15}.

The expression of factor VIII has also improved our understanding of this complex molecule and facilitates the characterization of specific mutants for the study of biochemical properties. Such studies have shown that the B domain of factor VIII can be deleted to yield factor VIII with functional procoagulant activity, both *in vitro* and *in vivo*¹⁶. Such B-domain deleted molecules are expressed more efficiently and may eventually yield forms of factor VIII that may be produced at lower cost. Further studies⁹ have increased our understanding of the structural

requirements for factor VIII function and may yield forms of factor VIII that are more easily administered and available for prophylactic use.

Finally, there is the possibility that gene replacement therapy will be achievable in the future. Retroviral-mediated transfer of B-domain deleted human factor VIII gene has recently been accomplished¹⁷. With developments in gene transfer techniques in humans, the development of ultrapure factor VIII by recombinant DNA technology may eventually be viewed as another step towards the cure for haemophilia A. □

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According to Dako Corporation, their **LSAB immunohistochemical staining kit** using streptavidin-biotin labelling techniques is four to eight times more sensitive than the avidin-biotin complex method (*Reader Service No. 102*). The kit is suitable for use with either mouse or rabbit antibodies: both react well with the biotinylated-link antibody provided, says Dako. Primary antibodies can either be provided by the user, or can be selected from Dako's comprehensive range of prediluted primary antibodies. The kit can be used to stain routinely fixed paraffin sections, smears, cryostat sections, imprints and cytocentrifuge preparations. Processing time is less than one hour, says Dako. The LSAB kit comes complete with all the reagents, accessories and instructions necessary to process at least 40 sections, says the company.

Researchers needing to make Na, K, Cl and CO₂ determinations may be interested in the **SYNCHRON EL-ISE electrolyte system**, which Beckman Instruments GmbH will be exhibiting in hall 4, stand 4C49 (*Reader Service No. 103*). EL-ISE is available in three models: the 2-channel analyser for Na and K determinations, the 3-channel Na, K and Cl analyser, and the



SYNCHRON EL-ISE: Beckman's electrolyte analyser.

4-channel Na, K, Cl and CO₂ analyser. Designed for use as either a stand-alone system or as a complement to a random access profiler, EL-ISE features automatic, manual, batch or STAT operating modes and a 40-sample turntable. Beckman emphasizes the high-throughput capabilities of the machine at greater than 100 total electrolyte panels per hour. The minimum sample requirements are 50 µl for serum, plasma and CSF and 60 µl for urine. Other features include disk-operated software available in six languages, storage and calculation of QC statistics for up to nine controls, preprogramming of up to six panels, selection of two Anion gap formulas and storage and recall of 120 samples.

ELISA kits

Using the new InterTest-6 ELISA kit for measuring human interleukin-6 (IL-6), Genzyme Corporation says results can be obtained in less than 24 hours and not days as with other methods (*Reader Service No.*



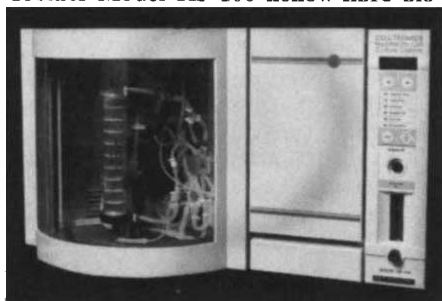
Genzyme's ELISA kit for measuring IL-6.

104). The InterTest-6 kit is, says Genzyme, more specific than existing bioassays, and is not affected by other constitutively produced cytokines — IL-1, IL-2, IL-3, IL-4, M-CSF, G-CSF, TNF-alpha or IFN-gamma — often present in test samples. Genzyme says the kit can be used to quantify natural or recombinant human IL-6 in serum, plasma or tissue culture supernatants in the range of 0.3-10 ng per ml. InterTest-6 has potential applications in the study of the inflammatory and immune response, progression and treatment of certain cancers, characterization of T-cell clones, and in the study of mononuclear cell release of IL-6 in peripheral blood. Kits are supplied with enough reagents for 96 determinations, including two standard curves. Genzyme Diagnostics will be on stand 5B42.

An ELISA kit for the detection of *Campylobacter pylori*-specific antibodies in serum or plasma is now available from Biometra biomedizinische Analytik GmbH (*Reader Service No. 105*). Using *C. pylori*-specific antigens immobilized to microwell plates and an enzyme-conjugated second antibody detection system, the *C. pylori* IgG titre can be calculated against standards of known IgG antibody concentration. The DM 494 (FRG) kit comprises a 96-well antigen-coated microplate with six double strips, all the necessary buffers, anti-human IgG enzyme-conjugated second antibody and six standards. The company will be exhibiting in hall 5, stand 5F39.

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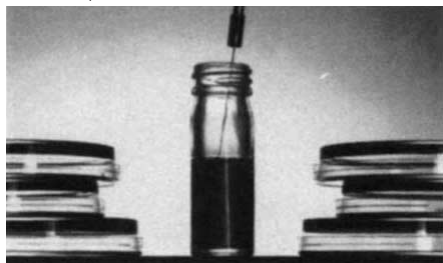
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Reader Service No.25

reactor from New Brunswick Scientific GmbH, who will be exhibiting in hall 3, stand 3B35 (*Reader Service No. 106*). The HF-100 benchtop unit consists of a pre-sterilized disposable hollow-fibre bio-reactor mounted in a temperature-controlled incubator that is regulated between 30-40 °C. There are two fluid transport systems — one for the circulation of medium and waste metabolite removal, and the other for the circulation of growth factor and the harvesting of cellular products. Using hollow-fibre technology, cells are cultured within the extracapillary spaces of the hollow-fibre cartridge, which has an area of 0.67 m². An automatic reverse-flow cycling action facilitates mixing of the medium, increases homogeneity of the culture and helps to prevent the formation of pH or nutrient gradients, says NBS. The user can, via the control panel, input set points and control process parameters such as cycle time, pump rates and CO₂ flow rates.

As an addition to Oxoid Ltd's comprehensive range of media, the company is offering **RVS broth for the selective enrichment of *Salmonellae*** (*Reader Service No. 107*). The RVS broth has been intro-



Oxoid's broth for *Salmonellae*.

duced as a modification of the Rappaport Vassiliadis (R10) broth, in that storage of the RVS broth produces no inhibitory effect due to pH drift on the growth of *Salmonellae*. RVS broth is available in 500-g quantities. Oxoid GmbH will be in hall 4, stand A22.

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Liquid handling

For precise **automated sample preparation** that will meet all laboratory liquid handling requirements, Quatro Biosystems recommends the Quatro 300 (*Reader Service No. 108*). Underpinning the flexibility



Automated sample preparation: Quatro 300 for liquid handling.

of the Quatro 300 is the menu-driven software, which will accept raw or processed data from external computers, readers or bar-code readers. This allows the user to create his or her own liquid handling routines for racks, tubes or microtitre plates. Alternatively, users can select from a range of preprogrammed routines. The Quatro 300 features high-speed probe operation, each of which can be programmed independently using the integral IBM-compatible computer. The XY positions of the probes are continuously monitored to maintain positional accuracy to within 0.125 mm, with sample carryover less than 0.005 per cent, says Quatro. The system can handle sample volumes between 5 and 1,000 µl and can process 475 tubes up to 17 mm in diameter or 10 microplates, or any combination thereof. Quatro Biosystems will be in hall 5, stand 5F07.

Visitors to hall 3, stand 3C53, can see Tecan GmbH's RSP 5000 Series **integrated laboratory system** for automated liquid handling, microplate reading and data management (*Reader Service No. 109*). With the modular base of the RSP 5000 Series, users can select from one- or two-armed sample processors, varying in size, hardware configuration and application software. Single, dual or multi-sampling tips can be moved in three dimensions by the robotic arms. The Teflon-coated sampling tips are equipped with liquid level detectors, and can be washed both inside and out during the pipetting routine. The system can handle sample/reagent volumes of 1 µl to 1.5 ml, says Tecan. Samples are analysed by the SLT microplate reader, which combines colourimetric technology with agglutination reading. The reader can make up to 25 readings per well and takes less than four seconds to read a plate, says the company. At the heart of the system is the LINCA software, which controls sample preparation and reading, while providing data handling capabilities.

Detection devices

Luminoskan is the new **microplate format luminometer** from Gruppo Flow SpA/Labsystems OY designed to streamline the measurement of luminescence light-generating reactions such as ATP, NAD(P) H/FMN, and hydrogen peroxide (*Reader Service No. 110*). Luminoskan has six measurement modes, including single measurement, peak value detection, integration over time, kinetics and rate reactions. Other features include four peristaltic dispensers for automatic reagent addition, either simultaneously with measurement or after a preset time-lag; plate mixer; and temperature control regulation between 20 and 43 °C. According to Gruppo Flow, Luminoskan can scan a plate in 25 seconds, giving one reading per well. The instrument has a spectral range of 200-680 nm and a sensitivity of 20 fmol ATP, says the company. Luminoskan can store up to 23 user-defined protocols of measurement parameters. The instrument can function as a stand-alone unit or can be computer-controlled. Luminoskan can be seen on Flow Laboratories' stand number A21 in hall 5.

The 1205 Betaplate liquid scintillation counter from Pharmacia Wallac Oy can now be used for high-throughput **scintillation proximity assays (SPA)** using the new T-tray attachment (*Reader Service No. 111*). Using the specially-designed 96-well plastic T-tray, all the dispensing and incubation steps can be carried out within the tray. Sample volumes can be up to 400 µl. The tray is then tape sealed and



Pharmacia's T-tray attachment for the 1205 Betaplate.

loaded into the counting cassette before transfer to the 1205 Betaplate counter: no liquid scintillation cocktail is required with SPA. Up to 20 cassettes (1,920 samples) can be processed at one time. Pharmacia says this procedure represents considerable savings in terms of time and consumables, and generates less solid and liquid radioactive waste than conventional set ups. Pharmacia LKB GmbH will be in hall 5, stand 5B17. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.